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Development of an Enzyme Immunoassay for Sulfamerazine in Milk and an Investigation into the Production of a Multiresidue Sulfonamide Enzyme Immunoassay

BY

Scott W. Garden

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

IN

Food Chemistry

Department of Food Science and Nutrition

EDMONTON, ALBERTA

Spring 1994

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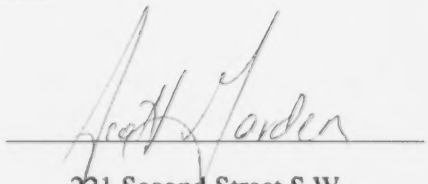

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Dr. Peter Sporns (Supervisor)

Dr. Pavel Jelen

Dr. Monica Palcic

Dr. Michael E. Stiles

Dr. Karl Kopecky

for Dr. Thomas A. Gill (External Examiner)

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To My Family

ABSTRACT

The thesis is presented as three papers examining the preparation and performance of polyclonal sulfamerazine (SMR) enzyme immunoassays (EIAs), and the attempted preparation of a monoclonal multiresidue sulfonamide EIA.

Competitor conjugates for the SMR EIAs were prepared using two different methods (azo and hemisuccinate) for linking SMR to bovine serum albumin (BSA). Hapten-protein molar ratios for the azo-linked conjugates were determined using a ^{14}C labeled sulfamethazine-BSA standard curve, *N*-chloroacetyl-L-tyrosine/*N*-α-acetyl-L-histidine standard curves, and amino acid analysis. Hapten amounts for the azo-linked conjugates were best estimated using the ^{14}C labeled sulfamethazine-BSA standard curve. Amino acid analysis indicated that hapten was predominantly linked to histidine residues of BSA. SMR hapten amounts for the hemisuccinate-linked conjugates were determined by acid hydrolysis, and subsequent sulfanilic acid quantification. An azo-linked conjugate had the lowest detection limit, requiring only 3.4 ppb of SMR to reduce absorbance in the assay by 50%.

The best azo- and hemisuccinate-linked competitor conjugates (hapten-protein molar ratios of 4.3 and 6.4, respectively) were compared with two commercial tests (Charm II assay and CITE Sulfa Trio) using milk samples from sulfonamide treated cows. Three cows were treated with oral doses of 3-Sulvit which contained SMR, sulfamethazine and sulfathiazole. Samples of the cows' milk were collected over 7 days, and 10 ppb cut-off dates determined for residual sulfonamide milk contamination. The SMR EIAs predicted the earliest cut-off dates, although closely followed by both commercial tests. The range of detection with the developed EIAs was 0.15-16.6 ppb of SMR.

During the attempted production of a multiresidue monoclonal sulfonamide EIA, hybridomas secreting IgM monoclonal antibodies (MAbs) were prepared. MAbs were collected from hybridomas grown in batch culture on serum-free media and purified using ammonium sulfate precipitation and dialysis. Quantification of MAb was performed using the Bio-Rad protein assay and ultraviolet spectrophotometric analysis. Hybridoma production rates ranged from 94 to 131 μg MAb/mL media. Unfortunately, detailed evaluation of purified IgM binding indicated a preference for lysine-modified conjugates rather than the conserved region of sulfonamides.

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LIST OF ABBREVIATIONS

1-BSA	amide-linked, N^1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide-bovine serum albumin conjugate
BSA	bovine serum albumin
BSA-FA	amide-linked, fumagillin-bovine serum albumin conjugate
B-M reaction	Bratton-Marshall reaction
^{14}CSMT	phenyl-ring-UL-^{14}C sulfamethazine
^{14}CSMT-BSA	azo-linked, carbon 14 labeled sulfamethazine-bovine serum albumin conjugate
^{14}CSMT-BSA(2)	azo-linked, carbon 14 labeled sulfamethazine-bovine serum albumin conjugate synthesized independently of the ^{14}CSMT-BSA conjugates
cpm	counts per minute
CZE	capillary zone electrophoresis
DCC	dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMSO	dimethylsulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EIA	enzyme immunoassay
FDA	Food and Drug Administration
GC	gas chromatography
GI	gastrointestinal
HAT medium	hypoxanthine, aminopterin and thymidine supplemented medium
HGPRT -	hypoxanthine guanine phosporibosyl transferase negative
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSFM	hybridoma serum-free medium
HSFM A1	hybridoma cell line "A1" grown in hybridoma serum-free medium
HSFM A6	hybridoma cell line "A6" grown in hybridoma serum-free medium
HT medium	hypoxanthine and thymidine supplemented medium
Ig	immunoglobulin
IR	infrared
1-LPH	amide-linked, N^1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide-*Limulus polyphemus* hemolymph conjugate

LPH	*Limulus polyphemus* hemolymph
MAb	monoclonal antibody
MS	mass spectrometry
NAH	*N*-α-acetyl-L-histidine
NCAT	*N*-chloroacetyl-L-tyrosine
NED	*N*-1-naphthylethylenediamine hydrochloride
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
OVA	ovalbumin
OVA-TS	amide-linked, N^1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide-ovalbumin conjugate
PABA	*p*-aminobenzoic acid
PBS	phosphate-buffered saline
PBST	phosphate-bufferd saline with Tween 20
PEG	polyethylene glycol
ppb	part per billion
ppm	part per million
RIA	radioimmunoassay
RPMI medium	Roswell Park Memorial Institute medium
RPMI(SR)	serum reduced Roswell Park Memorial Institute medium
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SDX	sulfadimethoxine
SMR	sulfamerazine
SMR-BSA(L)	lightly loaded, azo-linked, sulfamerazine-bovine serum albumin conjugate
SMR-BSA(M)	mediumly loaded, azo-linked, sulfamerazine-bovine serum albumin conjugate
SMR-BSA(H)	heavily loaded, azo-linked, sulfamerazine-bovine serum albumin conjugate
SMR-LPH	azo-linked, sulfamerazine-*Limulus polyphemus* hemolymph conjugate
SMR-NAH	azo-linked, sulfamerazine-*N*-α-acetyl-L-histidine conjugate
SMR-NCAT	azo-linked, sulfamerazine-*N*-chloroacetyl-L-tyrosine conjugate
SMR-SA	succinylsulfamerazine
SMR-SA-BSA(L)	lightly loaded, hemisuccinate-linked, sulfamerazine-bovine serum albumin conjugate

SMR-SA-BSA(M)	mediumly loaded, hemisuccinate-linked, sulfamerazine-bovine serum albumin conjugate
SMR-SA-BSA(H)	heavily loaded, hemisuccinate-linked, sulfamerazine-bovine serum albumin conjugate
SMT	sulfamethazine
SNM	sulfanilamide
SQX	sulfaquinoxaline
STZ	sulfathiazole
TEMED	tetramethylethylenediamine
TK^-	thymidine kinase negative
TLC	thin-layer chromatography
TMB	3,3',5,5'tetramethylbenzidine dihydrochloride
UV	ultraviolet
V_A	coefficient of variation of the absorbance values

Chapter 1:

INTRODUCTION

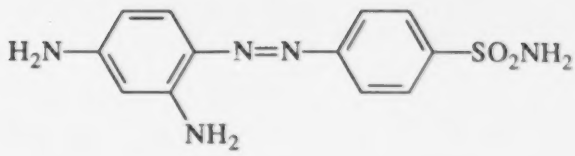
SULFONAMIDES

History of Sulfonamides

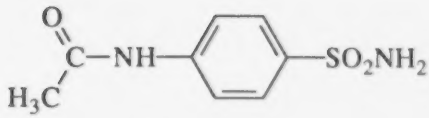
The introduction of sulfonamides in the 1930's marked the beginning of the modern antimicrobial era. The following historical review, taken from the publications of Lawrence (1946), Anand (1975), Bevill (1984) and Mandell and Sande (1990), highlights some of the significant events surrounding the discovery and evolution of sulfonamides.

The first sulfonamide derivative to be used therapeutically was the compound prontosil. Prontosil, a sulfonamide containing azo dye (Figure 1.1), was synthesized in 1931 by chemists Mietzsch and Klarer employed at I. G. Farben Industrie in Germany. The discovery of the chemotherapeutic properties of prontosil were the result of studies undertaken by the research director of I. G. Farben Industrie, Gerhard Domagk. In 1932, Domagk initiated the animal experiments which revealed the protective action of prontosil against streptococci and other infections in mice. From the animal experiments it was concluded that prontosil was active against streptococci *in vivo*, but that there was no observable *in vitro* activity. Although there was little doubt as to the clinical effectiveness of prontosil as a antimicrobial agent, questions were raised as to the nature of its activity.

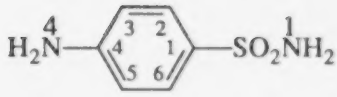
Late in 1935 researchers at the Pasteur Institute in France learned that the major urinary metabolitie following prontosil administration was acetylsulfanilamide (Figure 1.1). Trefouel and his colleagues postulated that cleavage of prontosil within the body liberated sulfanilamide (Figure 1.1), and it was sulfanilamide which conferred prontosil's antibacterial properties. Further research found that sulfanilamide was as effective as prontosil in protecting mice against lethal doses of streptococci. Although the antimicrobial properties of sulfanilamide were only then beginning to emerge, aspects of its chemistry had been known for almost three decades. Sulfanilamide was first synthesized in 1908 by Gelmo, a chemist working on azo compounds for the dye industry. The antibacterial properties of azo dyes containing the sulfonamide grouping (SO_2NH_2) had been recognized by Eisenberg in 1913, but animal studies at the time failed to produce any striking antibacterial results. Although prontosil had been patented by Mietzsch and Klarer, the eventual confirmation of sulfanilamide's role as the antimicrobial agent in prontosil negated any legal rights over its activity, since the sulfonamide segment of the drug was not patent protected. This allowed pharmaceutical companies the world over to synthesize sulfanilamide derivatives.



prontosil (sulfamidochrysoidine)



acetylsulfanilamide



sulfanilamide

Figure 1.1. Structures of Prontosil, Acetylsulfanilamide and Sulfanilamide.

Early sulfanilamide derivatives were synthesized in an attempt to produce compounds with greater clinical applications and to reduce the toxic effects encountered with the use of sulfanilamide. Synthesized in 1937, sulfapyridine was the first heterocyclic sulfonamide (Figure 1.2). Patients administered sulfapyridine, however, often suffered from the side effect of intense vomiting. Sulfathiazole, sulfadiazine, sulfamerazine, and sulfamethazine, synthesized between 1939 to 1942, (Figure 1.2) were found to be less toxic derivatives and produced higher therapeutic blood concentrations than sulfanilamide. Sulfaguanidine, synthesized in 1940, and succinylsulfathiazole and phthalylsulfathiazole synthesized in 1941, (Figure 1.3) were poorly absorbed from the gastrointestinal (GI) tract and proved valuable for the treatment of bacterial dysentery and pre-surgical sterilization of the gut.

The search for the "perfect" sulfonamide has led to the production and testing of 5500 compounds; however, only about 25 sulfonamides are used in modern clinical practice.

Chemistry of Sulfonamides

Sulfonamide is the general term used to describe derivatives of the compound sulfanilamide (Anand, 1975). Sulfanilamide or *p*-aminobenzenesulfonamide (Figure 1.1) is composed of a sulfonamide group (SO_2NH_2), linked through the sulfur atom to a benzene ring at carbon one, with the benzene ring linked to an amino group at carbon four. The nitrogen of the sulfonamide group is designated N^1 and is weakly acidic. The nitrogen of the aromatic amino group is designated as N^4 and acts as a weak base (Bevill, 1984). Sulfonamides are white or yellowish powders, and in their acid forms they are relatively insoluble in cold water and sparingly soluble in ethanol or acetone (Horwitz, 1981). Under basic conditions the weakly acidic N^1 can be converted to the sodium or potassium salt. The sulfonamide salts provide for significantly greater water solubility. Few sulfonamides produce a useful native fluorescence, although an adequately sensitive ultraviolet (UV) detection can be achieved at approximately 280 nm (Shepherd, 1991).

Mode of Action and Structurally Related Activity of Sulfonamides

The action of sulfonamides on both Gram-positive and Gram-negative bacteria is bacteriostatic. Bacteriostatic agents do not kill but only inhibit the growth of organisms, and once the agent is removed or its activity neutralized, the organism's growth should resume. What makes bacteriostatic agents effective is that the growth of the microbial infection is contained long enough for the body's own defense mechanisms to destroy the organisms (Brock, 1979).

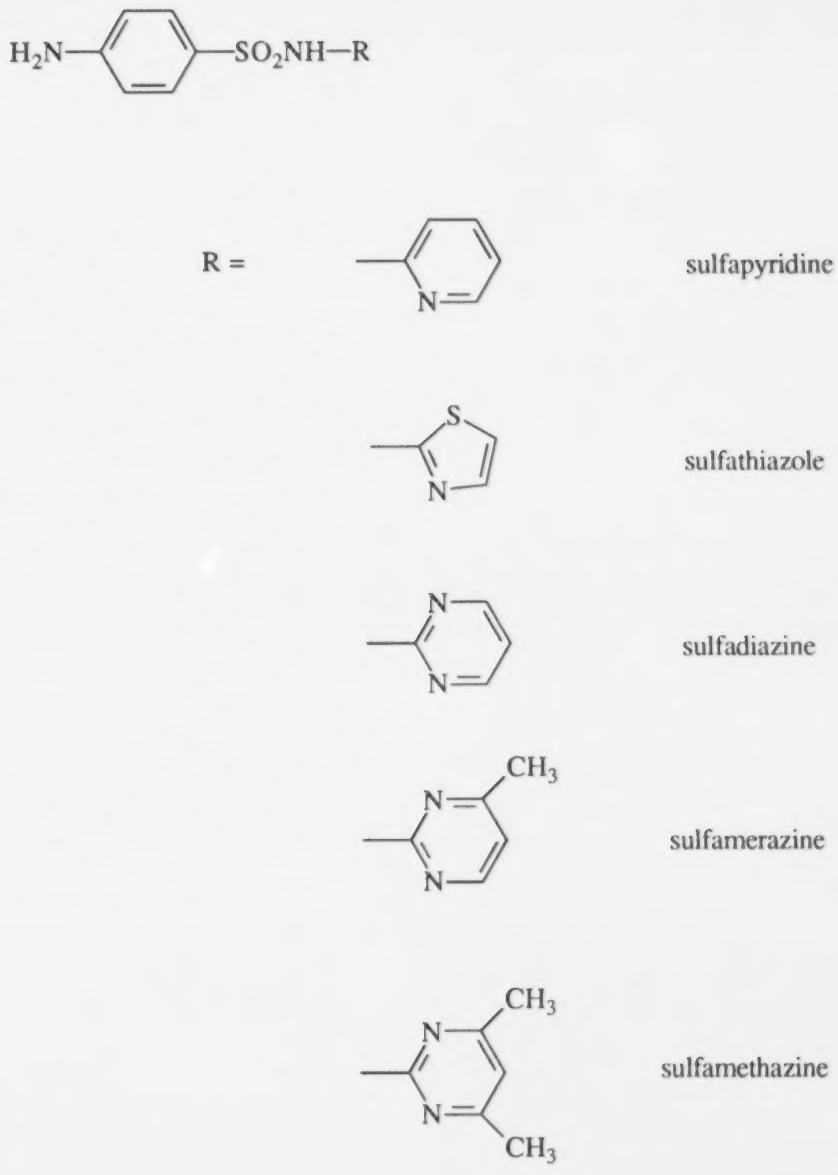
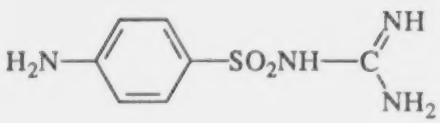
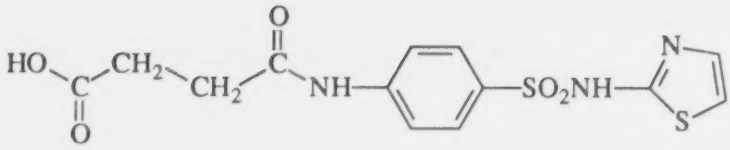


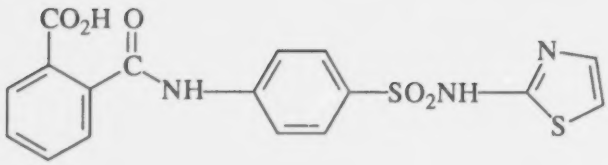
Figure 1.2. Structures of some Common Sulfonamides.



sulfaguanidine



succinylsulfathiazole



phthalylsulfathiazole

Figure 1.3. Structures of Sulfonamides Poorly Absorbed from the GI Tract.

The bacteriostatic effect of sulfonamides is derived from their analogous structure to *p*-aminobenzoic acid (PABA) (Figure 1.4) which is required by bacteria for the synthesis of folic acid (Mandell and Sande, 1990). Sulfonamides act as competitive antagonists of PABA by inhibiting the action of the enzyme dihydropteroate synthetase (Bevill, 1984). Dihydropteroate synthetase is responsible for the condensation of PABA and 2-amino-4-oxy-6-hydroxy-methyldihydropteridine pyrophosphate (dihydropteridine) to form dihydropteroate (Figure 1.4), an immediate precursor of dihydrofolate or folic acid (Anand, 1975). Sulfonamides may also prevent the synthesis of folic acid directly by inhibiting the enzymatic reaction between dihydropteridine and *p*-aminobenzoylglutamic acid. The inhibitory action of sulfonamides on folic acid synthesis ultimately results in a slowing down or prevention of the 1-carbon transferase reactions necessary for the biosynthesis of proteins, DNA and RNA (Anand, 1975).

Although sulfonamides have been synthesized with different chemical compositions, certain structural characteristics of sulfanilamide have been shown to be essential for sulfonamides to be antibacterially active. The para positioning of the aromatic amino group must be conserved because meta or ortho positional changes eliminate the PABA-related bacteriostatic activity of the sulfonamide (Lawrence, 1946). Conservation of the $-SO_2NH_2$ group of sulfanilamide is not essential as such, although it is important that the sulfur be directly linked to the benzene ring (Mandell and Sande, 1990). A few sulfonamides have been created through substitutions at the N^4 position (succinylsulfathiazole and phthalylsulfathiazole); however, in order for these sulfonamides to be bacteriostatic, *in vivo* cleavage of the N^4 substituent is required to liberate the amino group (Mandell and Sande, 1990). The majority of the sulfonamides are substituted at the N^1 position with substituents that have included alkyl, acyl, and aryl groups. However, the N^1 substituents with the greatest clinical applications have been the heterocyclic derivatives. The most clinically active five membered heterocyclic compounds are the thiazoles, oxazoles, isoxazoles, 1,3,4-thiadiazoles and pyrazoles. Important six-membered heterocyclic compounds have incorporated pyrimidine, pyridazine, and pyrazine groups, and of these the pyrimidines have provided the largest number of clinically useful agents (Anand, 1975).

The type of N^1 substituent is important in its effect on the ionization potential of this nitrogen. Bell and Roblin (1942) demonstrated that sulfonamides having a pKa in the range of 6-7.4 for the ionized nitrogen N^1 had the greatest bacteriostatic action. Bell and Roblin theorized that in this pKa range, the SO_2 group of the sulfonamide is able to increase its negative character by absorbing electron density from the ionized N^1.

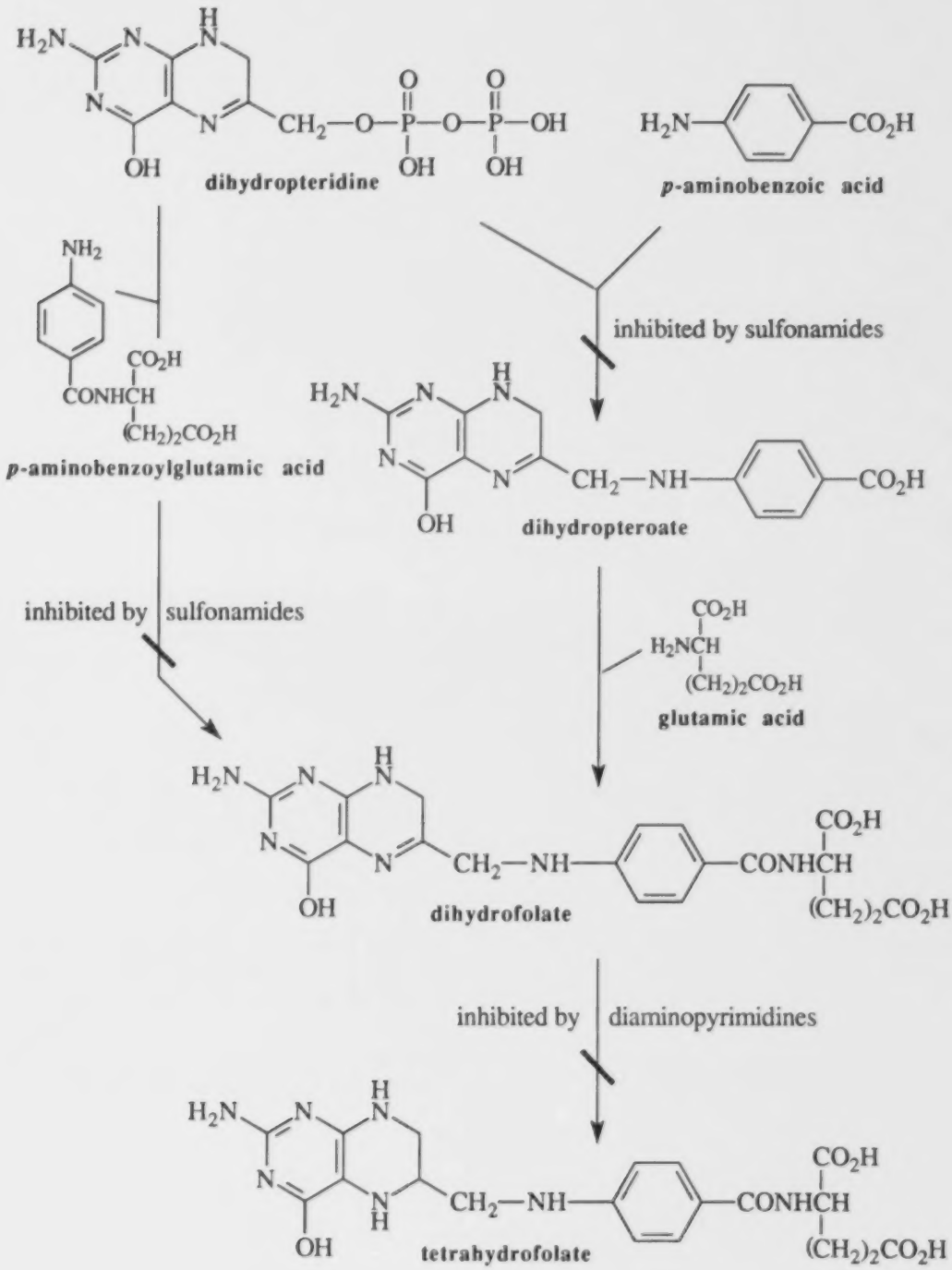


Figure 1.4. Enzymatic Synthesis of Tetrahydrofolate (adapted from Anand, 1975).

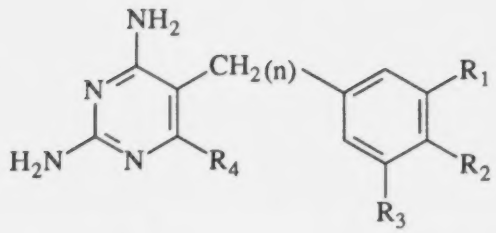
An increase in electron density allows the SO_2 group to more closely resemble the CO_2^- anion of PABA, which increases the bacteriostatic action of the sulfonamide.

In a review of the literature relating physicochemical properties of sulfonamides to their biological activity, Anand (1975) reports authors whose findings disputed the claims of Bell and Roblin (1942) of a relationship between the ionization of N^1 and an increased negative character of the SO_2 group. However, the authors referenced by Anand (1975), generally supported the claim of a correlation between the pKa of N^1 ionization and the bacteriostatic effect. It was concluded that the degree of sulfonamide activity relies on the balance between the sulfonamide's dissociation and hydrophobic character. The molecular (un-ionized) form of the sulfonamide is important since it relates to the sulfonamide's transport across cellular membranes, while the ionized form of the sulfonamide is required for enzyme interactions (Anand, 1975).

In the mid 1960's, the synergistic benefits of a combination therapy involving sulfonamides and a group of compounds known as diaminopyrimidines were recognized (Bevill, 1984). Diaminopyrimidines act in a sequential fashion with sulfonamides by inhibiting the function of dihydrofolate reductase, the enzyme responsible for the conversion of dihydrofolate (or folic acid) into the metabolically active tetrahydrofolate or folinic acid (Richards *et al.*, 1991) (Figure 1.4). It is tetrahydrofolate that is required for the 1-carbon transferase reactions in the biosynthesis of proteins, DNA and RNA (Anand, 1975). The list of diaminopyrimidines (Figure 1.5) includes diaveridine, methoprim, brodimoprim, tetroxoprim, ormetoprim, aditoprim, pyrimethamine, and the most commonly employed diaminopyrimidine, trimethoprim (Rehm *et al.*, 1986). Mammalian cells and bacteria which are able to use preformed folic acid are not affected by the sulfonamide/diaminopyrimidine mode of action (Mandell and Sande, 1990).

Administration, Absorption, Metabolism and Excretion of Sulfonamides

Sulfonamides can be administered via oral, intravenous, intramuscular, intraperitoneal, intrauterine and topical routes. In human therapy, oral and topical routes are most common, and in animals, oral, intravenous, intramuscular, and intraperitoneal are used (Bevill, 1984). For orally administered sulfonamides the primary site of absorption is the small intestine, with the exception of those poorly absorbed sulfonamides (i.e. sulfaguanidine, succinylsulfathiazole and phthalylsulfathiazole) created to treat localized bowel infections (Mandell and Sande, 1990). Once absorbed, sulfonamides are distributed throughout the body tissues as the native (unmetabolized) drug, bound to plasma proteins, or in various metabolic forms (Mandell and Sande, 1990).



	n	R1	R2	R3	R4
trimethoprim	1	OCH_3	OCH_3	OCH_3	H
diaveridine	1	OCH_3	OCH_3	H	H
methoprim	1	OCH_3	SCH_3	OCH_3	H
brodimoprim	1	OCH_3	Br	OCH_3	H
tetroxoprim	1	OCH_3	$O(CH_2)_2OCH_3$	OCH_3	H
ormetoprim	1	CH_3	OCH_3	OCH_3	H
aditoprim	1	OCH_3	$N(CH_3)_2$	OCH_3	H
pyrimethamine	0	H	Cl	H	CH_2CH_3

Figure 1.5. Structures of Diaminopyrimidines (adapted from Rehm *et al.*, 1986).

Sulfonamides are metabolized *in vivo*, mainly by the liver. The major metabolic pathway for most sulfonamides in animals is N^4-acetylation (Rehm *et al.*, 1986; Juskevich, 1987; Mandell and Sande, 1990) although N^4-acetylation only plays a minor role in birds (Vree and Hekster, 1985). The enzyme system involved in N^4-acetylation is the *N*-acetyltransferase-acetylcoenzyme A complex (Rehm *et al.*, 1986). N^4-acetylation is dependent on the N^1 substituent of the sulfonamide, and can vary widely between individuals and species (Vree and Hekster, 1985). Deacetylation of acetylated sulfonamides also occurs, but it has not been determined whether acetylation and deacetylation proceed through the same or separate enzyme systems (Rehm *et al.*, 1986).

A number of other pathways, besides N^4-acetylation, are available to metabolize and eliminate sulfonamides, although these pathways are generally used to a lesser extent. These pathways described in Horwitz (1981), Bevill (1984), Vree and Hekster (1985), Rehm *et al.* (1986) and Paulson (1987) include hydroxylation, glucuronidation, sulfatation, deamination, and O-dealkylation, and vary in importance between individuals and species. Hydroxylation is predominantly carried out by the cytochrome P-450 cluster, and results in the oxidation of aromatic ring systems and substituted methyl groups attached at the N^1 position. In glucuronidation, N^1-glucuronides are the primary species, though minor amounts of N^4-glucuronides and N-glucuronides in the N^1 substituent are formed. Sulfatation occurs through the action of sulfokinases which form sulfonamide-sulfate conjugates at the N^1 and the N^4 positions. Both glucuronidation and sulfatation can occur in sulfonamides that have been previously hydroxylated. Deamination occurs through an oxidative cleavage of the aromatic amino group from the sulfonamide, and unlike other sulfonamide metabolic reactions, decreases the polarity of the sulfonamide. O-dealkylation or cleavage of the heterocyclic ring involves the metabolic ether cleavage of aromatic ethers and substituted phenols. O-dealkylation products have been reported for sulfadimethoxine (Vree and Hekster, 1985; Rehm *et al.* 1986), but this form of metabolism is of only minor importance for sulfonamides. Figure 1.6 illustrates some metabolites of sulfamethazine as reported by Horwitz (1981).

The primary method of elimination of sulfonamides both in native drug and metabolite forms is in the urine, although small amounts may be excreted in the feces, bile, and milk (Mandell and Sande, 1990). The process of sulfonamide excretion involves the renal actions of glomerular filtration and tubular secretion, but the effectiveness of these processes may be hindered by active and passive reabsorption of sulfonamide in the renal tubules (Vree and Hekster, 1985).

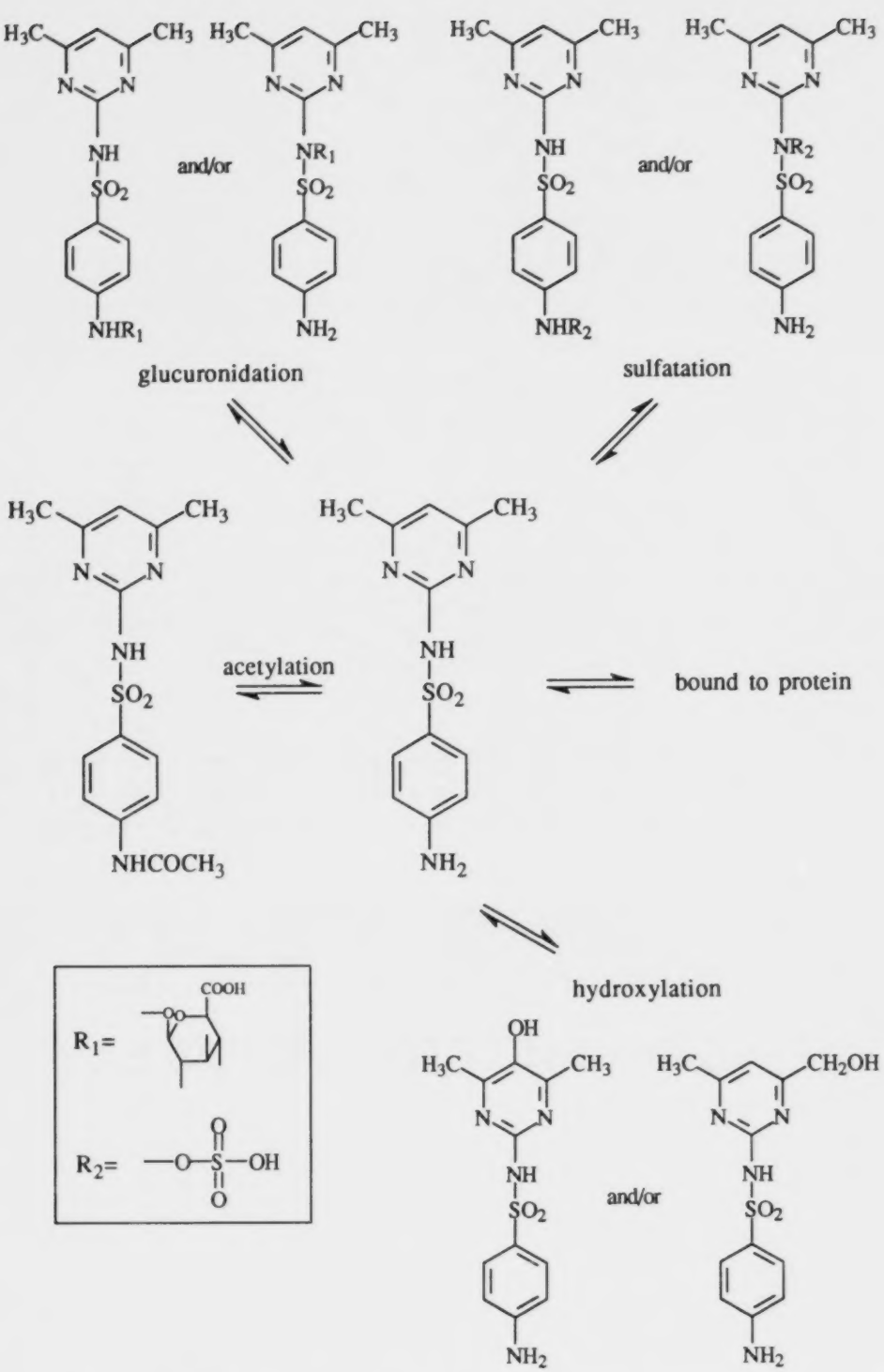


Figure 1.6. Metabolites of Sulfamethazine.

Factors promoting reabsorption are the inherent lipid solubility of the sulfonamides and their metabolites, and pH of the urine (Bevill, 1984). In acidic urine sulfonamides are ionized to a lesser degree than in alkaline urine. Because un-ionized sulfonamides are much more readily reabsorbed by the body than ionized sulfonamides, as the urinary pH decreases the renal clearance of sulfonamides also decreases (Bevill, 1984). Metabolic reactions which improve the water solubility of the sulfonamide aid in renal clearance. The body's production of polar sulfonamide metabolites generally serves to increase the water solubility of the parent compound, which enhances renal excretion (Rehm *et al.*, 1986).

Adverse Reactions to Sulfonamides

The overall incidence of adverse reactions following the administration of sulfonamides is about 5% (Mandell and Sande, 1990), and such reactions involve almost every system in the body (Anand, 1975). The types of reactions that are experienced can be separated into three general categories: toxicological, carcinogenic, and hypersensitivity. The following provides a brief description of some of the more common and/or serious reactions.

The toxicological effects of sulfonamides are primarily the result of disorders involving the urinary and hematopoietic systems (Lloyd and Mercer, 1984). The urinary system can be adversely affected through sulfonamide crystallization in the kidneys (crystalluria) resulting in subsequent lumbar pain, hematuria (blood in urine), and altered urine output (Bevill, 1984). Crystalluria occurs when glomerular filtration of sulfonamide exceeds the solubility product, resulting in deterioration of kidney function (Vree and Hekster, 1985). The incidence of crystalluria may be increased by the products of metabolism. Although acetylation increases the water solubility of sulfadiazine, sulfamerazine and sulfamethazine, it decreases the solubility of sulfanilamide and sulfathiazole (Rehm *et al.*, 1986). The risk of crystalluria may also be related to urinary pH. As stated earlier, sulfonamides being weak acids are more soluble in alkaline than acid urine. Therefore, a decrease in urine pH decreases sulfonamide solubility and promotes sulfonamide crystallization (Bevill, 1984).

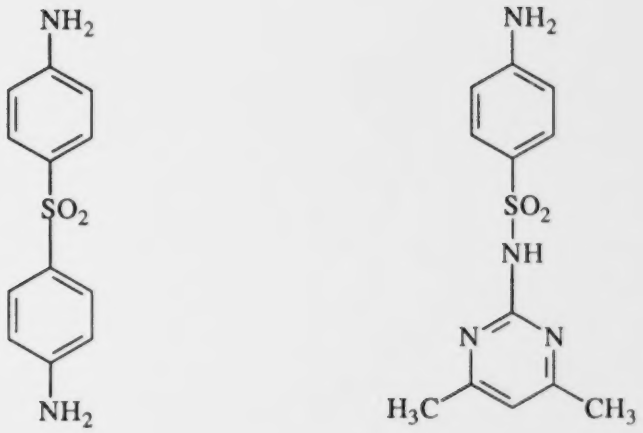
Adverse effects of sulfonamides on the hematopoietic system include acute hemolytic anemia (premature destruction of red blood cells), agranulocytosis (severe reduction of granulocytes), aplastic anemia (deficiency of formed elements in the blood), thrombocytopenia (reduction of platelets), and eosinophilia (increased number of eosinophils in the blood) (Huber, 1986). In most cases, the mode of action of sulfonamides in the hematopoietic disorders has not been clearly elucidated. Hemolysis, leading to acute hemolytic anemia, is possibly caused by either a sensitization reaction or

erythrocytic deficiency of glucose-6-phosphate dehydrogenase activity. Aplastic anemia probably results from direct toxic effects on the cellular components of the blood (Mandell and Sande, 1990).

The thought that sulfonamides may carcinogenic has resulted from their structural similarities to other potential carcinogens, and from direct studies of their actions. Structural similarities between dapsone, a suspected carcinogen (Griciute and Tomatis, 1980), and sulfamethazine (Figure 1.7) have led to reports expressing concern over sulfamethazine's carcinogenic potential (Charm *et al.*, 1988; Agarwal, 1992). In a study reported by Littlefield *et al.* (1990), Littlefield and colleagues found that sulfamethazine induced hyperplasia (increased cell production) and adenomas (glandular tumors) of the thyroid glands in rats. These results however, have been met with both support (Woodward, 1992) and skepticism (Shaw *et al.*, 1990).

Hypersensitivity reactions caused by sulfonamides can be the result of both topical and systemic sulfonamide administration. It is estimated that 3.5% of people given a therapeutic dose of sulfonamides will exhibit adverse skin and upper GI reactions (Rehm *et al.*, 1986), and 20% of people will demonstrate hypersensitivity reactions if they have received previous sulfonamide therapy (Lloyd and Mercer, 1984). The prevalence of skin reactions in humans ranges from 1.5 to 2%, (Huber, 1986) with one of the major manifestations being Stevens-Johnson syndrome (Anand, 1975). Stevens-Johnson syndrome is a serious, sometimes fatal inflammatory disease affecting children and young adults. Its symptoms include fever, blistering of the skin, ulcers of the lips, eyes, mouth, nasal passages and genitalia, pneumonia and joint pain (Anderson and Anderson, 1990). In a Food and Drug Administration (FDA) drug bulletin (1984), Stevens-Johnson syndrome was implicated in the death of three children, aged 4, 5, and 8, being treated with sulfamethazone/trimethoprim for a urinary tract infection, acute arthritis of the knee, and a middle ear infection, respectively.

Closely related to hypersensitivity are the idiosyncratic reactions. Idiosyncratic reactions are thought to occur from the conversion of parent drug into reactive metabolites, which either cause cell death and necrosis (localized tissue death) directly, or indirectly by initiating an immune response through binding to cellular proteins (Cribb and Spielberg, 1990). Idiosyncratic reactions include drug fever, hepatotoxicity, skin rash, blood dyscrasias (blood or bone marrow abnormalities), nephritis (kidney disease), and cardiotoxicity (Shear *et al.*, 1986). The reactive metabolites believed to be involved in idiosyncratic toxicity are the hydroxylamines, and the rate of formation of hydroxylamines in the body is dependent on the rate of acetylation (Cribb and Spielberg, 1990). In the



dapsone sulfamethazine

Figure 1.7. Structures of Dapsone and Sulfamethazine.

human population, slower acetylators are found to be at greater risk for iodiosyncratic reactions because their metabolism results in more sulfonamide available for oxidation to the hydroxylamine.

In most cases the adverse toxicological, hypersensitivity, and idiosyncratic reactions produced by sulfonamides can be stopped or reversed with the cessation of sulfonamide administration (Lloyd and Mercer, 1984; Rehm *et al.*, 1986; Cribb and Spielberg, 1990; Mandell and Sande, 1990).

Use of Sulfonamides in Animal Husbandry

Before the advent of antibiotics in the 1940's, sulfonamides were one of the few means available for treating infectious diseases in veterinary medicine (Roudaut and Moretain, 1990). Although there has been a reduction in use of sulfonamides in the therapy of human diseases, sulfonamides have persisted in veterinary medicine as economical and effective antimicrobials which can easily be administrated in feed and water (Bevill, 1984). Veterinary applications of sulfonamides can be divided into those of a therapeutic or subtherapeutic nature. Therapeutically, sulfonamides have been used to treat a wide range of infections in many livestock species including poultry, cattle, swine, sheep and fish (Steele and Beran, 1984). The use of sulfonamides in a subtherapeutic manner has been primarily a prophylactic measure to prevent the onset of disease, and to promote growth and efficient feed conversion (Franco *et al.*, 1990). The antimicrobial mode of action of sulfonamides in fighting bacterial infections has been well documented; however, the role that sulfonamides play in increased growth and feed efficiency is less clear.

The practice of adding antibiotics to commercial animal feeds was initiated by a 1949 publication which dealt with vitamin B_{12} supplementation of the diet of chicks (Jukes, 1984). The observed growth in chicks was greater than that which could be accounted for by the dietary addition of vitamin B_{12} alone. It was suggested that the presence of chlortetracycline in the fermentation products fed to the chicks resulted in their increased growth. Tindall *et al.* (1985) proposed the following mechanisms to explain the increased feed efficiency and growth which result in the antibiotic growth effect: (1) antibiotics can spare intakes of certain vitamins, minerals, amino acids, and energy for the animal; (2) antibiotics may selectively inhibit nutrient-using organisms; (3) antibiotics may increase feed and/or water intake; (4) antibiotics may inhibit the growth of microorganisms that produce toxins or toxic waste products; (5) antibiotics may kill or inhibit pathogenic organisms within the gastrointestinal tract; and (6) antibiotics may improve the digestion and absorption of certain nutrients. It should be noted that sulfonamides do not qualify as compounds that produce the "antibiotic growth effect", because they demonstrate no

nutrient sparing capabilities (Jukes, 1984). However, sulfonamides may promote growth by freeing energy for growth that the animal might have used to fight infection.

The perceived dangers of adding subtherapeutic levels of sulfonamides to animal feeds result from the possibility of residual contamination in animal products destined for human consumption or the emergence of drug resistant bacteria (Franco *et al.*, 1990). The adverse effects experienced as a result of sulfonamide administration are generally restricted to those patients receiving therapeutic doses of sulfonamides, although effects such as allergic reactions are not always dose related (Rehm *et al.*, 1986). However, Bevill (1984) found that there were no reports of acute or chronic toxicities, or hypersensitivities, associated with the ingestion of animal tissues containing sulfonamide residues. Concern over the risk of the emergence of sulfonamide resistant bacteria may be of greater relevance, because the levels of sulfonamide incorporated into animal feed are reportedly sufficient for selection of sulfonamide resistent enteric flora in animals ingesting such feed (Bevill, 1984). Enterobacteriaceae are of the greatest concern as they have plasmid-mediated resistance which could be passed from non-pathogenic to pathogenic organisms (Anand, 1975). The possibility exists that sulfonamide resistant organisms, cultivated in animals, could be tranferred to humans through the handling of animals or the consumption of animal products (Steele and Beran, 1984).

Residual sulfonamide contamination in cows' milk has recently been of concern in both the United States and Canada (Brady and Katz, 1988; Charm *et al.*, 1988; Collins-Thompson *et al.*, 1988; Larocque *et al.*, 1990). Sulfamethazine was generally found to be the contaminant, although it has never been permitted for intramammary use (Charm *et al.*, 1988; Larocque *et al.*, 1990). Some possible scenarios which could account for the presence of sulfamethazine in the milk are: the short-term administration of sulfamethazine to a few cows undergoing active treatment for disease; unintentional contamination by residual drug in the equipment used for feed preparation; or the routine use of sulfamethazine in lactating cows by a few delinquent dairy farmers (Larocque *et al.*, 1990). Over-the-counter products such as Sulfavite (Dominion Veterinary Laboratories Ltd., Winnipeg, MB) and 3-Sulfvit (Sanofi Santé Animale, Victoriaville, PQ) allow producers easy access to sulfonamide-containing preparations. Although package instructions warn against the use of these products in lactating dairy cattle, the warning is in very small print. Steps have been taken in an attempt to control the extent to which sulfonamides may be found in milk. A Joint Food and Agriculture Organization/World Health Organization expert committee on food additives (1989) recommended a 25 part per billion (ppb) maximum residue level for sulfamethazine in milk, and recently tolerances as low as 10 ppb for all sulfonamides were suggested (Zomer *et al.*, 1992). Ultimately though, controlling

the extent to which sulfonamides are present in the food we eat will depend, at least in part, upon the development of reliable and sensitive methods of sulfonamide detection.

Analytical Methods for the Detection of Sulfonamides

A wide range of analytical techniques has been explored for sulfonamide detection. The list includes colorimetric, chromatographic, microbiological, and immunological methods.

Colorimetric methods for sulfonamide detection have been based upon the reaction mechanism reported by Bratton and Marshall (1939) for the determination of sulfanilamide. In the Bratton-Marshall (B-M) reaction, the weakly basic aromatic amino group of the sulfonamide is diazotized with nitrous acid, and coupled with *N*-(1-naphthyl) ethylenediamine dihydrochloride to yield a colored azo compound. Colorimetric methods later developed by Houston and Umstead (1968) and Tishler *et al.* (1968) were based upon the B-M reaction. Limitations of colorimetric methods lay in their lack of sulfonamide specificity and extensive extraction and sample cleanup procedures. Also, there is the possibility of interference by primary aromatic amines (from sources other than sulfonamides) which may be present in body fluids and tissues from normal metabolism (Horwitz, 1981).

Chromatographic methods for the determination of sulfonamides in foods and feeds have been studied extensively (Horwitz, 1981). Coupled with an array of detection methods for identification and/or quantification, chromatographic techniques have been developed for sulfonamide detection using thin-layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary zone electrophoresis (CZE).

TLC methods for sulfonamide detection have been reported by Thomas *et al.* (1983), Wyhowski de Bukanski *et al.* (1988) and Unruh *et al.* (1990). A commercial TLC test, Sulfa On Site, has been produced by Environmental Diagnostics Inc. (Burlington, NC). Visualization techniques for TLC include UV absorption, the B-M reaction, and derivatization with *p*-dimethylaminobenzaldehyde, copper salts or other metals (Horwitz, 1981). A disadvantage of TLC methods is the need for complex sample extraction and preparation. Generally, applications of TLC have been for screening and qualitative analysis, however, a TLC method has been reported which is able to screen for eight sulfonamides at a 10 ppb level (Agarwal, 1992).

GC analyses for sulfonamides have been reported by Matusik *et al.* (1990), Takatsuki and Kikuchi (1990), and Carignan and Carrier (1991). Methods of detection for sulfonamides following gas chromatography include flame ionization detection, flame

photometric detection, electron capture detection, and mass spectral analysis. Although GC methodology offers low levels of detection and sulfonamide specificity when combined with mass spectrum determination, it suffers from complex sample extraction, cleanup and derivatization procedures (Horwitz, 1981). Also, sample analysis by GC requires expensive equipment and highly trained personnel for the operation of equipment and spectra interpretation, which may limit the practicality of its usage for certain applications.

Many HPLC methods have been described for multiresidue sulfonamide analysis (Aerts *et al.*, 1988; Ahmed and El-Gizawy, 1989; Horii *et al.*, 1990; Long *et al.*, 1990b; Roudaut and Moretain, 1990; Ikai *et al.*, 1991; Pleasance *et al.*, 1991; Wieling *et al.*, 1991; Agarwal, 1992; Reimer and Suarez, 1992; Zomer *et al.*, 1992; Abián *et al.*, 1993; Agarwal, 1993) and for individual sulfonamides (Houglum *et al.*, 1988; Weber and Smedley, 1989; Horie *et al.*, 1990; Long *et al.*, 1990a). Frequently, detection of sulfonamides by HPLC is accomplished by measuring UV absorbance in the range of 254-280 nm, although wavelengths as high as 290 nm and 310 nm have been used (Agarwal, 1992). More complex methodologies for the post-column detection of sulfonamides have included sulfonamide derivatization for fluorescence detection (Aerts *et al.*, 1988), ion-spray tandem mass spectometry (Pleasance *et al.*, 1991), thermospray mass spectrometry (Abián *et al.*, 1993), and the use of a microbial receptor (Zomer *et al.*, 1992). HPLC methods are able to identify individual sulfonamides while providing low detection limits. Although they do not require a pre-column derivatization, HPLC methods share many of the same disadvantages associated with GC methods. These include complex and time consuming sample extraction and cleanup in addition to capital equipment expenditures and personnel training.

Capillary zone electrophoresis is a relatively recent method used in sulfonamide detection (Wainright, 1990; Ackermans *et al.*, 1992; Ng *et al.*, 1992). Through the use of CZE and UV detection, individual sulfonamides can be identified and quantified once their mobility is known in an electrolyte solution. An advantage CZE, when compared to HPLC and GC, is the simple sample pretreatment, although expensive and complex equipment is still required for CZE. In a study by Ackermans *et al.* (1992), five sulfonamides were determined in pork meat extracts with a limit of detection of 2 to 9 parts per million. Sample preparation consisted of acetonitrile extraction of homogenized pork meat (stomacher apparatus for 5 min), followed by centrifugation (4000 x *g* for 10 min), and filtration (0.45 μm filter) prior to analysis.

Microbiological methods for sulfonamide detection have been based on two formats: the inhibition of microbial growth, or the use of sulfonamide-binding microbial receptors. The *Bacillus* genus has been a common choice as the indicator organism for

microbial inhibition tests and literature reports include the use of *Bacillus megaterium* (Read *et al.*, 1971), *Bacillus subtilis* (Bogaerts *et al.*, 1981) and *Bacillus stearothermophilus* (Charm *et al.*, 1988; Vermunt *et al.*, 1993). Many microbial inhibition tests have been commmercially produced, using *B. stearothermophilus* as the indicator organism, for the detection of sulfonamides in milk. These tests include the BR-Test "Blue Star" and BR-Test AS (Entorotox, Krefeld, Germany), the Delvotest-SP test (Gist-brocades Food Ingredients, Inc., King of Prussia, PA), and the Charm Farm test (Charm Sciences, Inc., Malden, MA). Fewer in number are the microbial receptor tests. The Charm Cowside and Charm II tests (Charm Sciences, Inc., Malden, MA) are the only two commercially produced microbial receptor assays, and like the microbial inhibition tests are marketed for sulfonamide detection in milk. The microbial-based tests are popular due to their ease of use, simple sample preparation, relative speed, multiple sulfonamide detection capabilities (although they cannot distinguish between individual sulfonamides), and low detection limits. Illustrating these advantages, the Charm II test uses milk samples directly, has an analysis time of 12-15 min per sample, and can detect a wide array of sulfonamides at a 10 ppb level. Recently, however, the ability of microbial based tests to provide accurate and consistent results for the detection of antimicrobials in milk has been questioned. Cullor (1992), Tyler *et al.* (1992) and Carlsson and Björck (1992) indicated that bacterial contamination, natural antimicrobial substances, high somatic cell counts, and free fatty acids present in milk may affect the reliability of microbial based tests.

Many immunological methods for the detection of sulfonamides have been developed (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; 1989; Singh *et al.*, 1989; McCaughey *et al.*, 1990; Sheth *et al.*, 1990; Sheth and Sporns, 1990; Dixon-Holland and Katz, 1991; Hoffmeister *et al.*, 1991; Sheth and Sporns, 1991; Assil *et al.*, 1992). The format used for the detection of sulfonamides in all cases was an enzyme immunoassay (EIA). Although some studies have had limited success in multiple sulfonamide detection (Sheth and Sporns, 1991; Assil *et al.*, 1992), development of EIAs has primarily centered upon the detection of sulfamethazine and a few other sulfonamides. Commercial EIA kits have been developed for the detection of sulfonamides in milk. These kits include the LacTek test (Idetek, Inc., Sunnyvale, CA), the CITE Sulfa Trio Test (IDEXX Laboratories, Inc., Westbrook, ME), Signal ForeSite Sulfamethazine (Smith Kline Beecham Animal Health, West Chester, PA), Agri-Screen ELISA (Neogen Corp., Lansing, MI), and the EZ-Screen test (Environmental Diagnostics, Inc., Burlington, NC). The EIA methodology for sulfonamide detection shares some of the same attributes as the microbial methods in ease of use, simple sample preparation, speed, and low detection capabilities. An advantage of EIAs is that they are specific for individual sulfonamides,

although some cross-reactivity is found between compounds which are very similar in structure (Fleeker and Lovett, 1985; Singh *et al.*, 1989; McCaughey *et al.*, 1990). Also, EIAs have performed reliably for the analysis of cows' milk under experimental endotoxin-induced mastitis conditions where microbial assays have been found to be unreliable (Tyler *et al.*, 1992).

ENZYME IMMUNOASSAYS

Introduction

An enzyme immunoassay is an assay procedure based on the reversible, noncovalent binding of an antibody to an antigen, using an enzyme-labeled antibody or antigen to quantify the system (Morris *et al.*, 1988). The use of enzyme labels in immunoassays was introduced independently in 1971 by Engvall and Perlmann, and Van Weeman and Schuurs (Rittenburg, 1990). The development of EIAs provided a viable alternative to the use of radioimmunoassays (RIAs) for food and agriculture diagnostics. RIAs were not perceived as entirely suitable for food and agriculture diagnostics since sample analysis by RIA required expensive and specialized equipment (scintillation counters), and special precautions for the handling and safe disposal of radioisotopes (Rittenburg, 1990). Conversely, enzyme labels used in EIAs are relatively inexpensive, and stable if properly stored. The conversion of a colorless substrate to a colored product by the enzyme label provides for sensitive and easily visualized endpoint interpretations (Rittenburg, 1990). Since their introduction, EIAs have developed as important analytical methods for the fast, simple and sensitive detection of a wide variety of compounds at exceedingly low levels. To understand how EIAs function for food analysis, it is necessary to discuss the major elements of the EIA system, including the antibody, antigen, enzyme label, and EIA format.

The Antibody

Antibodies belong to a structurally related family of glycoproteins known as immunoglobulins. Immunoglobulins (Igs) are produced by plasma cells of the B-lymphocyte lineage in response to foreign molecules (Harlow and Lane, 1988). Isotypes or classes of Igs which may be found in mammalian systems are IgG, IgM, IgA, IgE, and IgD. IgG is the principle Ig found in mammalian sera (Tijssen, 1985), and is commonly used in EIA experiments.

IgG is composed of four polypeptide subunits, two identical light chains and two identical heavy chains. The size of each light chain is approximately 25 kDa and each heavy chain is approximately 55 kDa (Harlow and Lane, 1988). Each light chain and heavy chain is composed of a variable region and constant region linked together by intrachain disulfide bridges. All four subunits in the completed IgG molecule are linked together by interchain disulfide bridges to form a symmetrical Y-shaped molecule of approximately 160 kDa in size (Harlow and Lane, 1988).

Other classes of Igs share some of the same structural characteristics as IgG. The size of the light chains is constant between classes of immunoglobulins, but the size of the heavy chain may vary in molecular weight from 50,000 to 80,000 depending on the isotype (Goding, 1986). IgG, IgE, and IgD are monomeric antibody structures consisting of one Y-shaped subunit. IgA can exist in a monomeric form, but commonly it is found in its secretory dimer form, composed of two Y-shaped subunits linked through their heavy chains (Coleman *et al.*, 1989). IgM is a pentamer and is composed of five Y-shaped subunits linked in a pinwheel structure (Coleman *et al.*, 1989).

Common to all Igs are antigen binding sites (paratopes) created where the variable regions of the light and heavy chains of the Ig intersect. IgG, IgE and IgD are bivalent antibodies with two identical antibody binding sites. IgA can have two (monomer form) or four (dimer form) antibody binding sites, while IgM has 10. The forces involved when the antibody binding site interacts with antigen include hydrogen bonding, charged group attractions, van der Waals forces and hydrophobic bonding (Coleman *et al.*, 1989).

Antibodies used in EIA may be polyclonal, monoclonal, or recombinant in nature. However, most assays are prepared using polyclonal or monoclonal antibodies (Lee and Morgan, 1993).

Polyclonal antibodies are isolated from the blood serum of an animal whose immune system has been stimulated by an immunogen. To produce the strongest immune response, any animal species other than that of the immunogen (or carrier protein used to synthesize the immunogen) may be used. The most commonly utilized animal hosts are goats, sheep and especially rabbits (Goding, 1986). Advantages of polyclonal antiserum are that it is relatively easy to produce and often contains a subpopulation of antibodies with fairly high affinities.

Monoclonal antibodies (MAbs) can be produced using mouse, rat or human lymphocytes, although the use of mouse lymphocytes is the most common (Campbell, 1984). MAbs are produced by hybridomas, a cell line which results from the fusion of B-lymphocytes from a suitably stimulated animal, and myeloma cells. Genetic material from both the B-lymphocytes and the myeloma cells is required for the production of viable hybridomas. Hybridomas acquire the ability for MAb synthesis and the utilization of the salvage pathway for nucleotide synthesis from the B-lymphocytes. The myeloma cells enable hybridomas to be grown continuously in cell culture (Tijssen, 1985).

The fusion of B-lymphocytes and myeloma cells is commonly performed using polyethylene glycol of molecular weight 1500 (Goding, 1986; Harlow and Lane, 1988) or 4000 (Campbell, 1984; Tijssen, 1985). The use of controlled culture conditions ensures that the few hybridomas produced during the fusion are preferentially selected for growth

from the large number of B-lymphocytes and myeloma cells present. B-lymphocytes are easily eliminated from cell culture, because if not fused to myelomas cells they naturally die under culture conditions. The myeloma cell line chosen for the fusion has been previously selected as being hypoxanthine guanine phosphoribosyl transferase negative (HGPRT $^-$) or thymidine kinase negative (TK$^-$). Being HGPRT $^-$ or TK$^-$, the myeloma cells lack the enzyme necessary for the utilization of the salvage pathway for the production of nucleic acid precursors (Tijssen, 1985). The salvage pathway provides the cells with the ability to produce nucleic acids when the de novo pathway is blocked (Figure 1.8). Therefore, since the myeloma cells only have the de novo pathway for the synthesis of nucleotides, when this pathway is blocked, the cells die. For 1 to 2 weeks after fusion, aminopterin is incorporated into the medium containing the B-lymphocytes, myeloma cells and hybridomas. Aminopterin is a folic acid antagonist which prevents the growth of myeloma cells by blocking de novo synthesis of nucleic acids. Aminopterin does not affect the growth of hybridoma cells that have acquired the genetic material from the B-lymphocytes that allows them to use the salvage pathway (Tijssen, 1985).

After elimination of the B-lymphocytes and myeloma cells, the hybridomas producing the desired MAbs are cloned. Hybridomas are most often cloned using some form of limiting dilution, where an entire culture is produced from the division of a single antibody-producing cell. This ensures a homogeneous population of antibodies. Following cloning, large-scale antibody production is commonly initiated. Large-scale quantities of MAbs from the cloned hybridoma may be produced through cell culture or the production of ascites tumors (Campbell, 1984).

For production of MAbs by cell culture, there are a variety of media and culture vessels from which to chose. Media commonly used include fetal bovine serum supplemented basal media (Roswell Park Memorial Institute medium 1640 (RPMI 1640) or Dulbecco's Modification of Eagles Medium (DMEM)), serum-free media (containing a defined protein content), or protein-free media. Culture vessels which may be employed include tissue culture flasks, roller bottles, spinner flasks, and hollow-fiber bioreactors. Any combination of media type or culture vessel can be used depending on the researcher's preference and hybridoma acceptability (Campbell, 1984).

The production of MAbs through ascites tumors involves the inoculation of the hybridomas into the intraperitoneal cavity of a mouse, where they multiply as a tumor. MAbs produced by the growing hybridomas are secreted into the ascitic fluid surrounding the tumor. The ascitic fluid containing the MAbs may be tapped from an anaesthetised mouse or drained from a dead one, and the antibody purified from the fluid as necessary (Campbell, 1984).

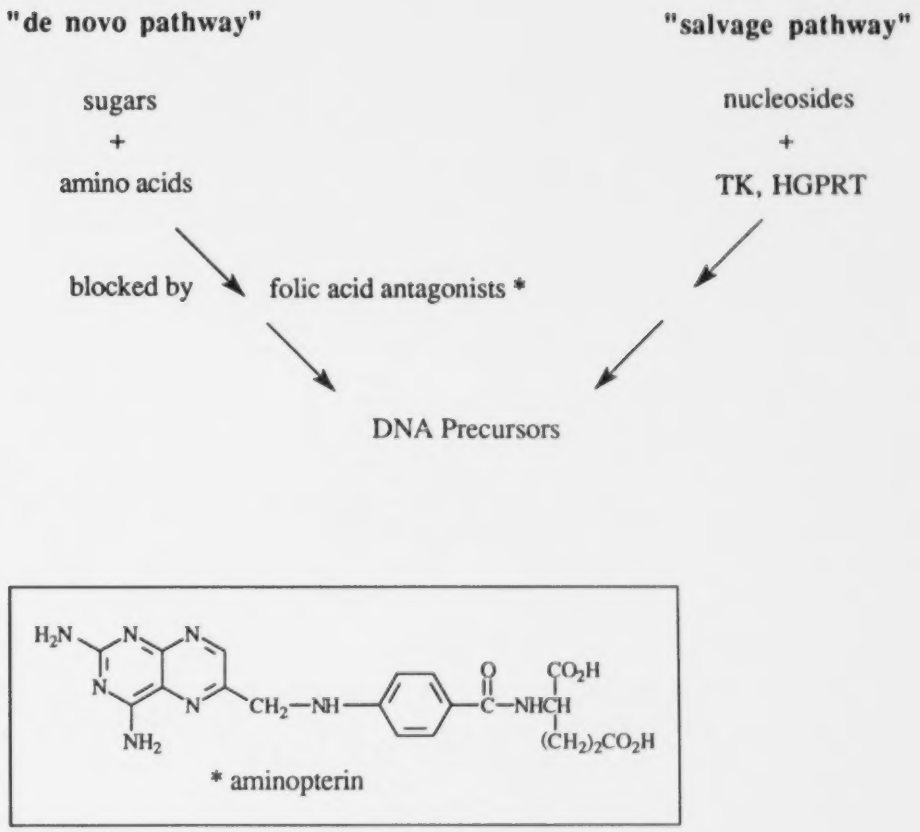


Figure 1.8. Two Pathways in Normal Cells to Synthesize DNA Precursors (adapted from Tijssen, 1985), and the Structure of Aminopterin.

The efficiency of B-lymphocyte and myeloma fusion is often low. Even in the most efficient fusions, only 1% of the initial cells are fused, and only 1 in 10^5 of the fused cells form viable hybrids (Harlow and Lane, 1988). Although more difficult and time consuming to produce, MAbs have the advantage over polyclonal antibodies in that they provide for an endless supply of antibodies with identical specificities and affinities.

Antigens, Immunogens, and Haptens

Park *et al.* (1987) defined an antigen as "a molecule capable of interacting with the antigen-combining site (hyper-variable region) of an immunoglobulin." An immunogen was described by Park and his colleagues as "a substance capable of eliciting a specific immune response manifested by the formation of specific antibodies and/or specifically committed lymphocytes. To induce an antibody response an immunogen must possess structurally and functionally distinct determinants for activation of B cells and T cells." The distinction is made between antigens and immunogens in that all immunogens are antigens, but all antigens are not necessarily immunogens. This differs from some other authors' definitions which consider the terms antigen and immunogen as interchangeable (Gazzaz *et al.*, 1992). Antigens contain one or more regions called antigenic determinants (or epitopes) which are able to specifically interact with a paratope (or antigen binding site) of the Ig. The size of the cavity of the antigen binding site may vary, but an epitope of approximately 5 to 7 amino acids in size (roughly 7 x 12 x 35 Å) can be accommodated (Gazzaz *et al.*, 1992). Macromolecules may contain many epitopes, and proteins usually have one epitope per 40 to 80 amino acids (Tijssen, 1985). Immunogenicity is often correlated with the size of the molecule, and molecules are generally not considered to be immunogenic if their size is less than 1000 Da (Gazzaz *et al.*, 1992). However, it should be noted that immunogenicity is not an intrinsic property of the size of the molecule and that some larger molecules may not be capable of stimulating an immune response (Tijssen, 1985). The term hapten is used to describe molecules (generally of a low molecular weight) which are able to provide one or more epitopes for antibody binding but are not themselves immunogenic.

To render haptens immunogenic they must be conjugated to a suitable immunogenic carrier. Most often the carriers are serum albumins from various species (Tijssen, 1985). Hapten-protein conjugation can be performed using the functional carboxylic, hydroxyl, sulfhydryl and amino groups which may be available on the hapten (Roe, 1991). Hapten densities in the range of 3 to 25 (Roe, 1991) or 8 to 25 (Erlanger, 1980; Tijssen, 1985) moles of hapten per mole of protein have been suggested for immunization or successful

EIAs. Too few or too many hapten molecules per carrier molecule, generally results in a poor immunological response (Tijssen, 1985).

The Enzyme Label

Enzymes used as labels in EIAs generally have the following characteristics: a high turnover number, the ability to be easily purified, stability, easily coupled to proteins and haptens, and the availability of substrates which produce easily measured products (Rittenburg, 1990). A typical turnover rate for an enzyme label is at least 10^6 molecules of substrate into product, per molecule of enzyme per minute, at ambient temperature (Rittenburg, 1990). The most commonly chosen enzyme labels for EIAs are horseradish peroxidase (HRP), alkaline phosphatase and ß-galactosidase, and of this group HRP is the most frequently used (Porstmann and Porstmann, 1988). HRP is a 44 kDa glycoprotein, and EIAs commonly employ the "C" isozyme of the horseradish *Armoracia rusticana* (Tijssen, 1985). Popular water soluble substrates for HRP include 2,2'-azinodi(ethylbenthiazoline)sulfonate, 3,3',5,5'tetramethylbenzidine (TMB) and *o*-phenylenediamine (Harlow and Lane, 1988). These substrates are added to EIAs as colorless solutions, and in the presence of hydrogen peroxide (H_2O_2), are converted to colored products by HRP. An example of the mechanism HRP uses to convert the colorless substrate TMB to the colored product 3,3',5,5'-tetramethyl-1,1'-diphenoquinone-4,4'-diimoniumion is given in Figure 1.9. In EIAs the enzyme is conjugated to either an antibody or an antigen, which provides the means of detecting the formation of an immune complex (commonly antigen-antibody or antigen-antibody-antibody). Enzyme conjugates may be made between the enzyme and a primary antibody, a secondary antibody (whose specificity is for the Ig class of the primary "detection" antibody) or the antigen being analyzed, depending on the EIA format.

Formats of Enzyme Immunoassays

Solid supports used in EIA's include glass, silica, nylon, sepharose, polyacrylamide, agarose, cellulose, cyanogen bromide-activated paper and nitrocellulose paper (Campbell, 1984; Rittenburg, 1990). The most popular EIA supports, however, are polyvinyl or polystyrene in the form of a 96 well microtiter plate. For the analysis of hapten molecules such as sulfonamides, the most commonly used EIA formats are the direct and indirect competitive immunoassays. To achieve quantifiable color development, competitive direct EIAs may use either enzyme labeled antigen/hapten, or an enzyme-labeled primary "detection" antibody (Morris *et al.*, 1988; Rittenburg, 1990).

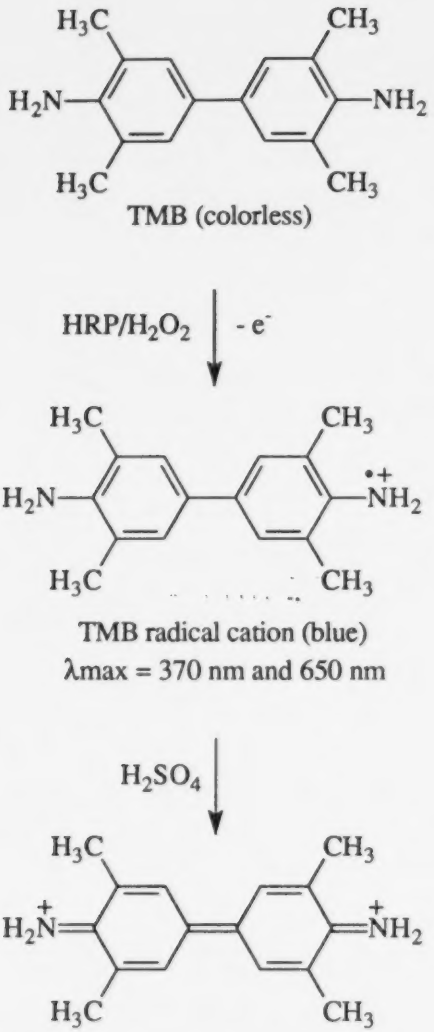


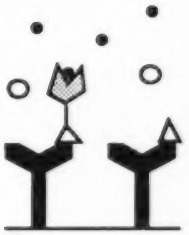
Figure 1.9. HRP and Sulfuric Acid (H_2SO_4) Conversion of Colorless TMB Substrate to Colored Product (adapted from Porstmann and Porstmann, 1988).

Color development is achieved in the competitive indirect EIA by use of an enzyme-labeled secondary antibody whose specificity is for the Ig class of the primary "detection" antibody (Rittenburg, 1990). Figures 1.10 and 1.11 and 1.12 depict the basic steps involved in each of the variations (enzyme-labeled hapten or antibody) of the competitive direct EIA, and the competitive indirect EIA. Color development in each competitive EIA system is inversely related to the amount of hapten in the sample being analyzed. Therefore, as the amount of free (or sample) hapten added to the system increases, the amount of color developed in the system decreases.

- well of microtiter plate coated with antibodies

- addition of free and enzyme-labeled hapten which compete for antibody binding sites

- conversion of enzyme substrate to colored product by hapten-bound enzyme



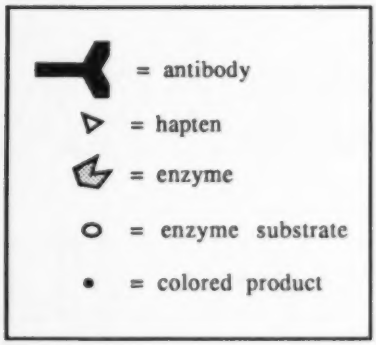
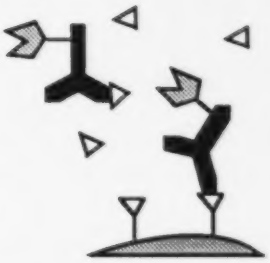


Figure 1.10. Competitive Direct EIA (enzyme-labeled hapten).

- well of microtiter plate coated with hapten-conjugated carrier protein

- addition of enzyme-labeled antibodies and free hapten resulting in a competition between bound and free hapten for enzyme-labeled antibodies' binding sites



- conversion of enzyme substrate to colored product by antibody-bound enzyme

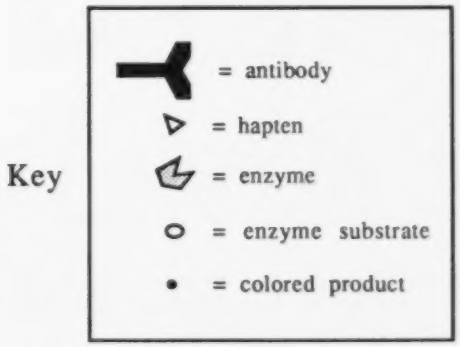
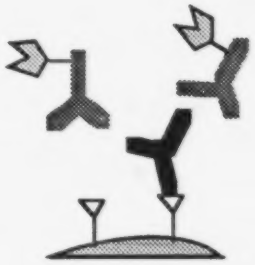


Figure 1.11. Competitive Direct EIA (enzyme-labeled antibody).

- well of microtiter plate coated with hapten-conjugated carrier protein

- addition of primary antibodies and free hapten resulting in a competition between free and bound hapten for the primary antibodies' binding sites

- addition of enzyme-labeled secondary antibodies specific for the primary antibody



- conversion of substrate to product by secondary antibody-bound enzyme

Key

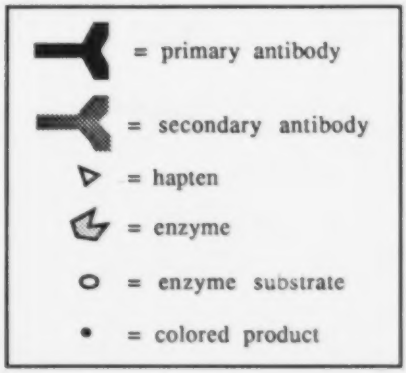


Figure 1.12. Competitive Indirect EIA.

THE THESIS

Objectives

Recently, there has been a growing concern over residual sulfonamide contamination in animal products destine for human consumption. One way of addressing this concern is to provide adequately sensitive tests for sulfonamide analyses. The three papers of this thesis explore the use of EIAs for the sensitive and reliable detection of sulfonamides. The first two papers describe the preparation and evaluation of EIAs for the analysis of SMR in milk. SMR has become a recent concern as a potential contaminant of cows' milk, and to date, there have been no reports of the development of a SMR EIA. The third paper of this thesis describes an investigation into the production EIA using a monoclonal antibody with multiresidue sulfonamide detection capabilities. To date there have been no reports of the development of such a potentially valuable EIA utilizing such an antibody.

The first paper describes the preparation and characterization of competitor conjugates for SMR EIAs. The objectives of this paper were the inter- and intra-comparisons of six competitor conjugates prepared with two hapten-protein linking methods. Inter-comparisons of the competitor conjugates were performed using conjugates prepared with an azo- or a hemisuccinate-link for covalently bonding SMR to BSA. The intra-comparisons of competitor conjugates were performed using three conjugates synthesized with the same hapten-protein linking method, but each having a different hapten-protein molar ratio. For both the inter- and intra-comparisons of competitor conjugates, it was necessary to explore many approaches for accurately determining hapten-protein molar ratios since no previous methodologies for such conjugates had been developed. Once the hapten-protein molar ratios were known, SMR detection limits for each of the competitor conjugates could be determined.

The second paper explores the use of competitor conjugates developed in the first paper, for the practical application of detecting SMR residues in milk samples from sulfonamide treated cows. For this study, a competitor conjugate was chosen from each of the linking methods (azo and hemisuccinate) which had the lowest SMR detection limits. Milk samples from the sulfonamide treated cows served to evaluate the performance of the competitor conjugates relative to each other, and also to commercially available sulfonamide tests (Charm II assay and CITE Sulfa Trio test).

The final paper is an investigation into the production of a monoclonal, multiresidue sulfonamide EIA. In the first two papers, polyclonal antiserum used in EIAs had a specificity for a single sulfonamide, SMR. The primary objective of the third paper was to

produce a MAb having multiresidue sulfonamide detection capabilities using the structural region common to all sulfonamides. Other objectives of this study involved the examination of different protocols which may be used during MAb preparation. These included the use of serum-free medium and batch culture for MAb production, ammonium sulfate precipitation and dialysis for MAb purification, and the Bio-Rad protein assay and UV spectrophotometric methodology for MAb quantification.

It should be noted that during the various studies in this thesis, all experiments involving the use of animals were approved by the appropriate animal welfare committees at the University of Alberta. In addition, every effort was made by myself and others to limit any discomfort experienced by the animals.

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Chapter: 2

AN ENZYME IMMUNOASSAY FOR SULFAMERAZINE. 1. Evaluation of Methods for the Determination of Hapten-Protein Molar Ratios*

* A version of this chapter has been accepted for publication.
Garden and Sporns (1994). Journal of Agricultural and Food Chemistry.

INTRODUCTION

Sulfonamides are a group of compounds of considerable importance in the treatment of microbial infections, both in humans and animals. From the numerous sulfonamides which have been synthesized, 18 are of relevance for use in veterinary medicine (Rehm *et al.*, 1986). One of these, sulfamethazine (SMT), is commonly employed for the therapeutic and subtherapeutic treatment of livestock. Studies indicating that SMT can have serious implications for human health (Franco *et al.*, 1990; Littlefield *et al.*, 1990; Shaw *et al.*, 1990; Woodward, 1992) have led to concern over the prospect of residual contamination when SMT is used in animals whose products are destined for human consumption. To control SMT residues, tests have been developed exploiting the sensitivity and specificity of the immunoassay (Fleeker and Lovett, 1985; Singh *et al.*, 1989; McCaughey *et al.*, 1990; Dixon-Holland and Katz, 1991). However these tests are specific for SMT, and will not detect other important sulfonamides such as sulfamerazine (SMR). SMR is very similar in structure to SMT, and therapeutically SMR may be administered alone (Pleasance *et al.*, 1991), or in preparations with other sulfonamides. Although there are many commercially available immunochemical tests for the detection of SMT (Medina *et al.*, 1992), none have been produced for SMR.

This study describes the preparation of a competitive indirect enzyme immunoassay (EIA) for SMR. During the development of the EIA, parameters such as the method of hapten linkage, and the hapten-protein molar ratio, were examined to determine their affect on the assay's detection capability.

EXPERIMENTAL

Instrumentation

The EIA analyses were performed in 96-well, Immulon 2, flat-bottomed microtiter plates from Dynatech Laboratories Inc. (Chantilly, VA) with Linbro nonsterile, acetate plate sealers manufactured by ICN Biomedicals, Inc. (Costa Mesa, CA) to prevent evaporation. Absorbance measurements were made using a Model EL 309 microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). Spectra/Por 2 membrane tubing (12,000-14,000 molecular weight cut-off) from Spectrum Medical Industries Inc. (Los Angeles, CA) was used for dialyzing the conjugates, and a Virtis 5L freeze-drier (The Virtis Company Inc., Gardiner, NY) for lyophilization. Spectrophotometric analyses were performed using an HP 8451A Diode Array Spectrophotometer from Hewlett-Packard Canada Ltd. (Mississauga, ON). Solvents were removed from samples using a Büchi Rotavapor from Fisher Scientific (Ottawa, ON). PE SIL G/UV TLC plates with polyester backing obtained from Whatman Ltd. (Maidstone, England) and a Model UVS-54 lamp (254 nm light emission) produced by Ultra Violet Products Inc. (San Gabriel, CA) were used for thin-layer chromatography (TLC). Carbon 14 radioactive decay was measured in 20 mL plastic scintillation vials from Fisher Scientific (Ottawa, ON), using EcoLite+ scintillation cocktail from ICN Biomedicals Inc. (Irvine, CA) in an LS 1801 Liquid Scintillation Counter from Beckman Instruments Inc. (Mississauga, ON).

Nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared (IR) spectroscopy, and microanalyses were all performed by the laboratories of University of Alberta Chemistry Services. NMR spectra were measured using a Bruker WH-400 instrument and MS spectra using a Kratos AEI MS-50 (high resolution, electron impact ionization) for exact mass determination. Microanalyses were measured using a Perkin-Elmer 240 CHN analyser for carbon, hydrogen and nitrogen. Sulfur was determined using barium perchlorate titration after the sample had been burnt in an oxygen filled flask. IR spectroscopic analyses were measured as a Nujol mull using a Nicolet 7199 FT-IR spectrophotometer.

Amino acid analyses were carried out at the University of Alberta Biochemistry Department on a Beckman 6300 system, with a cation exchange resin and a post-column ninhydrin detection system. Integration of peaks was performed using Beckman System Gold data analysis software.

Reagents

Sulfamerazine (SMR), sulfamethazine (SMT), sulfamethazine-phenyl-ring-UL-^{14}C (^{14}CSMT), bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-chloroacetyl-L-tyrosine (NCAT), *N*-α-acetyl-L-histidine (NAH), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB), ammonium sulfamate, thimerosal, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium nitrite was obtained from Fisher Scientific (Ottawa, ON) and sulfanilic acid from Terochem Laboratories Ltd. (Edmonton, AB). Succinic anhydride was supplied by BDH Chemicals (Edmonton, AB) and anhydrous *N,N*-dimethylformamide by Aldrich Chemical Co. Inc. (Milwaukee, WI). *N*-1-naphthylethylenediamine dihydrochloride (NED) was obtained from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI). Urea peroxide and goat anti-rabbit peroxidase-conjugated antibodies were supplied by Calbiochem Co. (San Diego, CA).

All other chemicals were of reagent grade or better, and solutions were prepared using water purified by a Millipore Milli-Q system (Millipore Corp., Millford, MA).

Preparation of Succinylsulfamerazine (SMR-SA)

To prepare SMR-SA (reaction (1), Figure 2.1), 1.33 g (5.05 mmol) of the acid form of SMR, 2.67 g (26.7 mmol) of succinic anhydride, and 0.0588 g (0.481 mmol) of DMAP, were dissolved in 15 mL of pyridine. The solution was heated at 55°C for approximately 3 h in an oil bath, and the endpoint of the reaction determined by TLC. The TLC solvent contained acetone: n-heptane: methanol: ammonia: n-butanol in a ratio of 72:21:9:10:10 (v/v) (Bregha-Morris, 1979).

After cooling, the pyridine containing SMR-SA was transferred to a separatory funnel with 20 mL of water. This solution was washed 3 times with 10 mL of methylene chloride. The combined methylene chloride washings were then washed 3 times with 10 mL additions of 0.1 N hydrochloric acid, then once with 10 mL of water. The methylene chloride extract was dried over anhydrous sodium sulfate and the solution was filtered through Whatman no. 4 filter paper into a round bottom flask. The methylene choride was co-distilled with toluene on a rotary evaporator with water bath (45-55°C) until a beige powder (SMR-SA) was obtained. The SMR-SA powder was then recrystallized from water with a 46% yield of purified crystalline material.

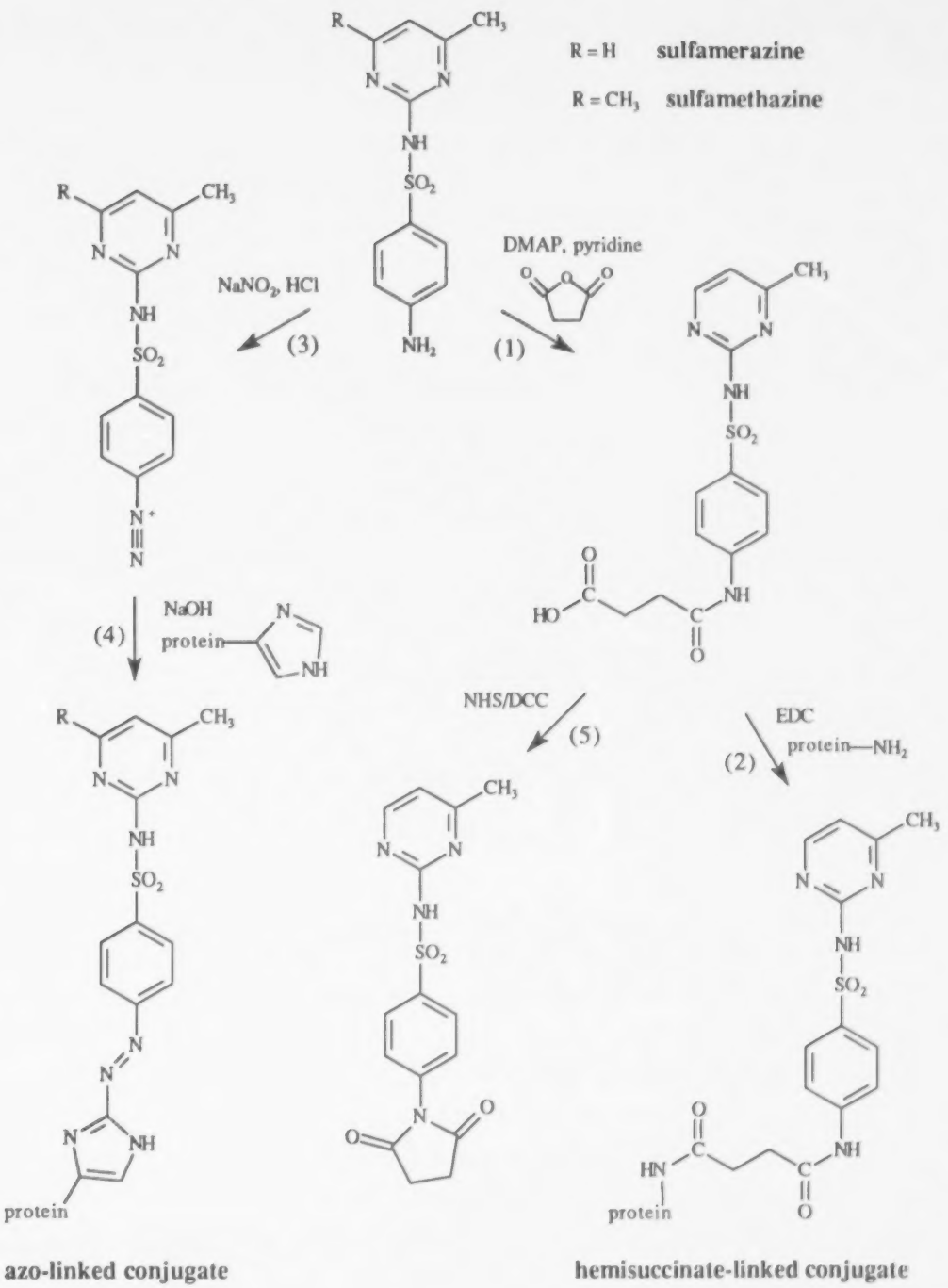


Figure 2.1. Preparation of Azo- and Hemisuccinate-linked Conjugates.

The structure of the purified SMR-SA powder was confirmed by NMR, MS and IR spectroscopy. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.88 (very br s, 2 H, N*H*), 10.34 (s, 1 H, COO*H*), 8.29 (d, 1 H, 5.1 Hz, Ar *H*), 7.90 (d, 2 H, 8.5 Hz, Ar *H*), 7.72 (d, 2 H, 8.5 Hz, Ar *H*), 6.88 (d, 1 H, 5.1 Hz, Ar *H*), 2.57 (t, 2 H, 6.0 Hz, C*H*$_2$), 2.51 (t, 2 H, 6.0 Hz, C*H*$_2$), 2.27 (s, 3 H, C*H*$_3$). Exact mass $(M\text{-}HSO_2)^+$ *m/z* 299 (2.42%) calcd. for $C_{15}H_{15}N_4O_3$: 299.1144 (measured 299.1148). Exact mass $(M\text{-}H_2SO_3)^+$ *m/z* 281 (52.78%) calcd. for $C_{15}H_{13}N_4O_2$: 281.1038 (measured 281.1040). The IR spectrum of SMR-SA showed 2 strong absorption bands at 1720.95 and 1673.50 cm^{-1} which were not present in the IR of SMR, indicating the hemisuccinate linkage had been formed.

Preparation of Hemisuccinate-linked Competitor Conjugates

A general procedure was used for the synthesis of the hemisuccinate-linked competitor conjugates (reaction (2), Figure 2.1). The synthesis of the lightly loaded, hemisuccinate-linked, sulfamerazine-bovine serum albumin conjugate (SMR-SA-BSA(L)) involved dissolving 10.4 mg (0.0285 mmol) of SMR-SA and 11.0 mg (0.0573 mmol) of EDC in 1 mL of anhydrous *N,N*-dimethylformamide. A molar ratio of approximately 2:1 (EDC:SMR-SA) was used in the synthesis of all hemisuccinate conjugates. The SMR-SA/EDC solution was added dropwise to a rapidly stirring solution composed of 75.12 mg of BSA, 1 mL of water, and 20 μL of 6 N sodium hydroxide. Six hours later, 10.7 mg of EDC (approximately 2 times the initial molar concentration of SMR-SA) was added to the reaction mixture which was left to stir overnight at room temperature. Table 2.1 provides the molar concentration of reactants used for the medium and heavily loaded conjugates, SMR-SA-BSA(M) and SMR-SA-BSA(H), respectively.

The next day the SMR-SA-BSA conjugates were transferred from the reaction flask to Spectra/Por 2 membrane tubing with washings of 8 M urea, and dialyzed against 1 L of 8 M urea for 16 h. The dialysis tubing and contents were then transferred to a 4 L beaker containing 50 mM ammonium bicarbonate and dialyzed for 12-16 h, followed by dialysis against 25 mM ammonium bicarbonate for a further 12-16 h. Finally, the conjugate was removed from the dialysis bag and freeze-dried. These dialysis and lyophilization procedures were common to all sulfonamide-protein conjugates.

Preparation of Azo-linked Competitor Conjugates

A general procedure was used for the synthesis of all the azo-linked conjugates. The synthesis involved the production of an SMR diazonium ion (reaction (3), Figure 2.1), which was further reacted with a protein to form an azo-linked conjugate (reaction (4), Figure 2.1).

Table 2.1. Concentrations of Reactants used in the Synthesis of Azo- and Hemisuccinate-linked Conjugates

Azo-linked Conjugates			Hemisuccinate-linked Conjugates		
Conjugate	Diazonium ion (mmoles)	Protein (mg)	Conjugate	SMR-SA (mmoles)	Protein (mg)
SMR-BSA(L)	0.0267	257.5	SMR-SA-BSA(L)	0.0285	75.12
SMR-BSA(M)	0.0522	207.0	SMR-SA-BSA(M)	0.0832	75.12
SMR-BSA(H)	0.376	255.8	SMR-SA-BSA(H)	0.101	53.4
SMR-LPH	0.0261	106.9			

During the synthesis of the lightly loaded, azo-linked, sulfamerazine-bovine serum albumin conjugate (SMR-BSA(L)), 7.65 mg (0.0267 mmol) of SMR was dissolved in 1 mL of 3.5 N hydrochloric acid. This was followed by the addition of 0.4 mL of a 1% aqueous sodium nitrite (0.0580 mmol) and then 19.6 mg (0.172 mmol) of ammonium sulfamate, mixing the solution well between each addition. This produced the SMR diazonium ion solution required for protein conjugation.

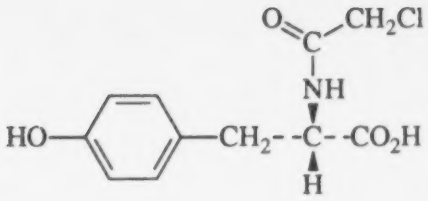
The SMR diazonium ion solution was added dropwise (with rapid mixing) to 257.5 mg of BSA dissolved in 4 mL of PBS (pH 7.3) in a 10 mL pear-shaped flask. The solution was left to mix for 5 min, then 20 drops of 6 N sodium hydroxide were added from a pasteur pipet to raise the pH of the solution to greater than eight. The flask and contents were wrapped in foil and left to stir overnight at 4°C, then dialyzed and freeze-dried in the same manner as the hemisuccinate-linked competitor conjugates.

For the synthesis of immunogen (SMR-LPH) and the medium and heavily loaded competitor conjugates (SMR-BSA(M) and SMR-BSA(H), respectively), the amount of SMR diazonium ion and BSA used are given in Table 2.1.

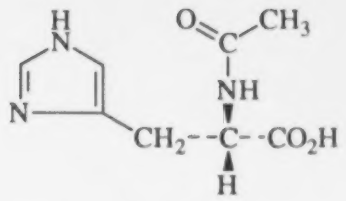
Preparation of the *N*-Chloroacetyl-L-tyrosine (NCAT) and *N*-α-Acetyl-L-histidine (NAH) Standard Curves

For the NCAT standard curve, SMR dilutions were prepared using 0.1 N sodium hydroxide and ranged from 3.510 to 0.4712 μmol SMR/mL. For the NAH standard curve, SMR dilutions were prepared with 0.1 N sodium hydroxide and ranged from 175 to 23.6 nmol SMR/mL.

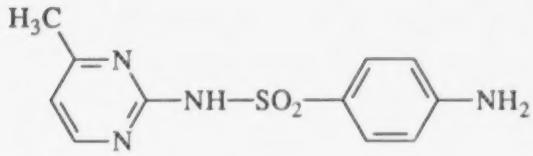
The NCAT and NAH standard curves were produced by reacting the SMR dilutions under the following conditions. A 0.75 mL aliquot of a SMR dilution was added to 0.75 mL of 3.5 N hydrochloric acid and vigorously mixed. Next, 0.23 mL of a 1% aqueous solution of sodium nitrite was added, followed by 0.35 mL of a 0.8% aqueous solution of ammonium sulfamate, mixing the solution well between each addition. Depending on the standard curve, the next addition was either 0.75 mL of a 13.52 mg NCAT/mL 0.1 N sodium hydroxide (52.46 μmol/mL) solution or 0.75 mL of a 10.34 mg NAH/mL 0.1 N sodium hydroxide (52.43 μmol/mL) solution. Finally, 0.75 mL of 6 N sodium hydroxide was added, and the solutions left stirring overnight at 4°C. This reaction produced an azo link between either SMR and NCAT (SMR-NCAT) or SMR and NAH (SMR-NAH), depending on the standard curve (Figure 2.2). A blank solution was prepared by substituting 0.75 mL of 0.1 N sodium hydroxide for the SMR dilution in the first step of the reaction.



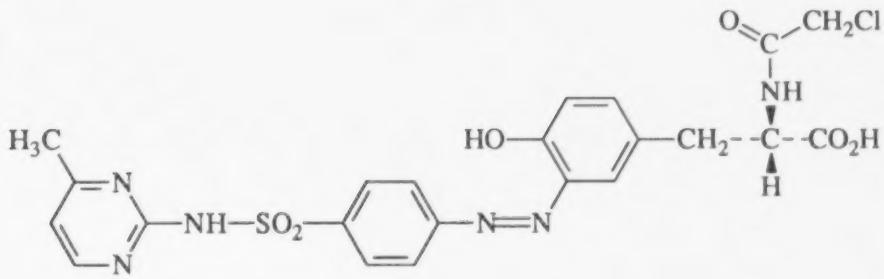
N-chloroacetyl-L-tyrosine (NCAT)



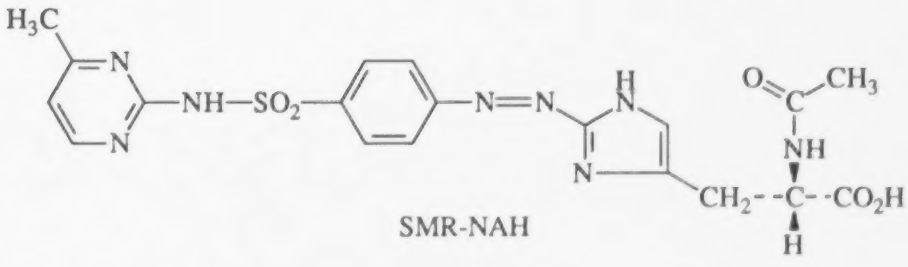
N-α-acetyl-L-histidine (NAH)



sulfamerazine (SMR)



SMR-NCAT



SMR-NAH

Figure 2.2. Structures of NCAT, NAH, SMR, SMR-NCAT and SMR-NAH.

The following day the absorbances of all the standard curve solutions (SMR-NCAT and SMR-NAH) were measured at 492 nm (the absorbance maximum of the SMR-NCAT solutions) and at 436 nm (the absorbance maximum of the SMR-NAH solutions). The absorbance values at 492 nm and 436 nm for both SMR-NCAT and SMR-NAH solutions were plotted against the molar concentration of the SMR dilution. From these plots linear relationships were obtained, with each having a coefficient of determination (r^2) of 0.98 or greater. The slopes of the lines were used to calculate equation (1) and (2), which represent the molar concentrations of SMR-NCAT ($M_{SMR\text{-}NCAT}$) and SMR-NAH ($M_{SMR\text{-}NAH}$), respectively.

$$M_{SMR\text{-}NCAT} = [12.0(A_{492nm}) - 3.83(A_{436nm})] \times 10^{-4} \quad (1)$$

$$M_{SMR\text{-}NAH} = [5.53(A_{436nm}) - 3.98(A_{492nm})] \times 10^{-5} \quad (2)$$

An accurately weighed sample of each of the azo-linked competitor conjugates was dissolved in 0.6 N sodium hydroxide in a 10 mL volumetric flask. Absorbance readings for each of the azo-linked conjugate solutions were measured at 492 nm and 436 nm. The number of moles of SMR/mole protein calculated using equations (1) and (2) are reported in Table 2.2.

Preparation of Azo-linked, Carbon 14 Labeled Sulfamethazine-Bovine Serum Albumin (^{14}CSMT-BSA) Standard Curve

A "hot stock" was prepared by dispersing 50 µCi of lyophilized ^{14}CSMT in 200 µL of PBS (adjusted to pH 8.2). "Hot and cold stock" was prepared by combining a 50 µL aliquot of the "hot stock" with 250.84 mg of SMT in a 5 mL volumetric flask (diluted to volume with 0.1 N sodium hydroxide). BSA (1.5093 g) dissolved in 10 mL of PBS (pH 7.3) was used as the "BSA stock" solution. The "hot and cold stock" (50.17 mg SMT/mL 0.1N sodium hydroxide) and "BSA stock" (150.93 mg BSA/mL PBS) were used to synthesize each of the ^{14}CSMT-BSA conjugates for the standard curve.

The synthesis of the ^{14}CSMT-BSA conjugates was performed following the same series of reactions (reactions (3) and (4), Figure 2.1) used to prepare the azo-linked conjugates. To prepare the diazonium ion for the most heavily loaded ^{14}CSMT-BSA conjugate, 0.4 mL of "hot and cold stock" and 0.4 mL of 3.5 N hydrochloric acid were combined in a test tube. This was followed by the addition of 0.5 mL of 1% sodium

Table 2.2. Hapten-Protein Molar Ratios Determined for Azo-linked Competitor Conjugates

Method of Analysis		Azo-linked Competitor Conjugates (moles hapten/mole protein)		
		SMR-BSA(L)	SMR-BSA(M)	SMR-BSA(H)
NCAT/NAH standard curves	$M_{SMR\text{-}NCAT}$[a]	8.1	17.6	90.0
	$M_{SMR\text{-}NAH}$[b]	1.3	3.0	5.5
	total	9.4	20.6	95.5
NAH standard curve (A_{436nm})		1.5	3.6	7.9
^{14}CSMT-BSA standard curve		4.3	10.0	22.2
Amino Acid Analysis (glycine)	azotyrosines	0.3	0.6	nd[c]
	azohistidines	2.1	7.7	nd
	total	2.4	8.3	
Amino Acid Analysis (arginine)	azotyrosines	0.2	-0.1	nd
	azohistidines	2.0	7.3	nd
	total	2.2	7.3[d]	

[a] values for moles hapten/mole protein were calculated for each conjugate using the molar concentration of SMR derived from equation (1), and the known concentration of protein used for absorbance measurements.

[b] values for moles hapten/mole protein were calculated for each conjugate using the molar concentration of SMR derived from equation (2), and the known concentration of protein used for absorbance measurements.

[c] nd denotes that results were "not determined" for this conjugate

[d] negative value not considered in total

nitrite, then 16.6 mg of ammonium sulfamate, mixing the solution well between each addition. To prepare other ^{14}CSMT-BSA conjugates for the standard curve, the ratio of ^{14}CSMT to BSA was varied by decreasing the volume of "hot and cold stock" that was used. Sodium hydroxide (0.1 N solution) was added before the addition of 3.5 N hydrochloric acid, as required to keep the total volume in the test tube constant from conjugate to conjugate.

The ^{14}CSMT-BSA diazonium ion solution from the test tube was added dropwise, with rapid mixing, to a pear-shaped flask containing 1.0 mL of the "BSA stock" solution and 1.3 mL of PBS (pH 7.3). Finally, 0.4 mL of 0.6 N sodium hydroxide was added to the reaction mixture in the pear-shaped flask to raise the pH above eight. The flask was then wrapped in foil and left to stir overnight at 4°C.

The next day the contents of the pear-shaped flask containing the ^{14}CSMT-BSA conjugate were transferred to Spectra/Por 2 membrane tubing, and dialyzed and freeze-dried as described for the hemisuccinate-linked competitor conjugates. After lyophilization, a precisely weighed portion of the conjugate was dissolved in 0.6 N sodium hydroxide in a 10 mL volumetric flask. This solution was used for spectrophotometric analysis and for radioactive decay measurements.

The ^{14}CSMT-BSA standard curve was created by plotting the absorbance value at 430 nm (A_{430nm}/mg of protein conjugate/mL 0.6 N sodium hydroxide) versus the moles of SMT/mole BSA (determined by radioactive decay) for each of the ^{14}CSMT-BSA conjugates that were synthesized. From the ^{14}CSMT-BSA standard curve, equation (3) was derived ($r^2 = 0.99$). Using equation (3), values were calculated for the moles of sulfonamide/mole protein for the azo-linked SMR-BSA competitor conjugates, and reported in Table 2.2.

$$m = \frac{A_{430nm}}{0.123} \qquad (3)$$

m = moles of sulfonamide per mole protein

Amino Acid Analysis of the Azo-linked Competitor Conjugates

To hydrolyse the azo-linked competitor conjugates for analysis, approximately 0.1 mg of the conjugate was dissolved in 5 drops of boiling hydrochloric acid (containing 0.1% phenol) in a 10 x 75 mm borosilicate culture tube. The top of the tube was elongated

in an oxygen flame, the tube evacuated using a vacuum pump, and the neck of the tube sealed. The tube was placed in a 100°C oven overnight. The next day the top of the tube was scored and removed, and the tube placed in a vacuum dessicator with sodium hydroxide overnight. The tube contents were dissolved in 100 µL of 0.2 M citric acid buffer (pH 2.2) and samples taken for analysis on the Beckman 6300 system.

The retention times and the response factors of each of the amino acids were confirmed by analyzing a set of amino acid standards before the analysis of the hydrolysed protein conjugates. To provide a protein standard for comparison, BSA was hydrolysed, and the amino acid composition measured in the same manner as the azo-linked competitor conjugates. Data from the amino acid analysis were examined, and the modifications to each of the azo-linked competitor conjugates (using BSA as a standard) were calculated using equation (4).

$$R = \left(\frac{[A]_{BSA} - [A]_{conj}\left([C]_{BSA} / [C]_{conj} \right)}{[A]_{BSA}} \right) x\ N \qquad (4)$$

R = number of azo-linked amino acid residues/BSA

A = tyrosine or histidine

C = glycine or arginine

N = tyrosine or histidine molecules/molecule of BSA (tyrosine, 20; histidine, 17)

$[\ ]_{BSA}$ = nanomoles of the amino acid in BSA

$[\ ]_{conj}$ = nanomoles of the amino acid in the azo conjugate

From equation (4), estimates were made of the number of azotyrosine and azohistidine residues (using glycine and arginine as internal standards) for each of the azo-linked conjugates. The results are reported in Table 2.2.

Preparation of the Sulfanilic Acid Standard Curve

The sulfanilic acid standard curve was prepared using a modified Bratton-Marshall (B-M) reaction similar to that described by Low *et al.* (1989). Aqueous dilutions of sulfanilic acid used to prepare the curve ranged from 50 to 5 µg/mL. An aliquot of each sulfanilic acid dilution (1 mL) was added to 4 mL of 6 N hydrochloric acid, and 1 mL of each of these sulfanilic acid/hydrochloric acid solutions was used in the modified B-M

reaction. The 0.8% aqueous NED solution was prepared the day of the assay, as the solution blackened with time.

Modified B-M Reaction. To 1 mL of each of the sulfanilic acid/hydrochloric acid solutions was added 200 µL of 0.2% aqueous dilution of sodium nitrite, and the solution vigorously mixed. This was followed by the addition of 200 µL of 3.3% aqueous ammonium sulfamate solution, 500 µL of 98% ethanol, and 200 µL of the 0.8% aqueous NED, mixing the solutions well between each of the additions. After 15 min in the dark, the absorbance of the solutions was measured at 554 nm. A standard curve was created by plotting the A_{554nm} against the molar concentration of the sulfanilic acid in each dilution. The slope of the curve was 5082 (absorbance units/M) with $r^2 = 1.00$.

Hydrolysis of Hemisuccinate-linked Competitor Conjugates

Each of the hemisuccinate-linked competitor conjugates was hydrolysed, and the hapten-protein molar ratios determined, under the following set of reaction conditions.

An aqueous solution was prepared containing between 2 and 7 mg of a freeze-dried hemisuccinate-linked competitor conjugate, in a 10 mL volumetric flask. A 1 mL sample was taken from the volumetric flask and added to 4 mL of 6 N hydrochloric acid. This solution was refluxed for 2.5 h. Once cooled, 1 mL of the competitor conjugate hydrolysate was used in the modified B-M reaction in place of the sulfanilic acid/hydrochloric acid dilutions. For each hemisuccinate competitor conjugate, an absorbance value at 554 nm was obtained, and this value converted to a molar concentration of SMR-SA in the conjugate using the sulfanilic acid standard curve. By accounting for the weight of SMR-SA for each of the competitor conjugates, the molar concentration of BSA in the conjugates could be calculated. The molar ratios of SMR-SA to BSA for each of the hemisuccinate-linked competitor conjugates are reported in Table 2.3.

Citric Acid Buffer

A 0.1 M aqueous solution of citric acid monohydrate and a 0.1 M aqueous solution of sodium citrate were mixed in proportions to produce a 0.1 M citrate buffer solution with a pH of 4.0.

Table 2.3. Hapten-Protein Molar Ratios Determined for Hemisuccinate-linked Competitor Conjugates

Method of Analysis	Hemisuccinate-linked Competitor Conjugates (moles hapten/mole protein)		
	SMR-SA-BSA(L)	SMR-SA-BSA(M)	SMR-SA-BSA(H)
Sulfanilic Acid Curve	6.4	18.6	22.0

Phosphate-buffered Saline (PBS and PBST) Solutions

The PBS solution was prepared by dissolving sodium chloride (9.0 g), disodium hydrogen phosphate (1.108 g), potassium dihydrogen phosphate (0.3 g) and thimerosal (0.1 g) in 1 L of water. PBS, prepared without thimerosal, was autoclaved and used in the preparation of the immunogen (SMR-LPH). To prepare PBST, 0.5 g (0.05%) of Tween 20 was added to the PBS solution prior to dilution to volume with water. The pH of the PBS or PBST was adjusted to 7.3 with sodium hydroxide or hydrochloric acid as needed.

Polyclonal Antibody Antiserum Preparation

Limulus polyphemus hemolymph (LPH) was chosen for the preparation of the immunogen (SMR-LPH), as it was a protein foreign to the immune system of the experimental animals. The SMR-LPH conjugate had a hapten-protein molar ratio of 8.4, which was calculated using the ^{14}CSMT-BSA standard curve. This was within the range of 8 to 25 hapten molecules/BSA as prescribed for strong antibody titers by Erlanger (1980). For the production of antiserum, two 12 week old, female, Flemish Giant x Dutch Lop Ear rabbits were each injected with a water-in-oil emulsion composed of 1 mg SMR-LPH dissolved in 1 mL of PBS (pH 7.3) and 1 mL of Freund's complete adjuvant. The rabbits were injected subscapularly (2 x 0.5 mL) and intramuscularly (2 x 0.5 mL). Twenty-eight days later, the rabbits were immunized again following the same protocol as for the initial injections except Freund's incomplete adjuvant replaced Freund's complete adjuvant. On day 41 (from the initial injections) the first bleed was taken for analysis (10 mL from the large artery of the ear of each rabbit). On day 64, both rabbits were immunized once again in the manner described earlier, and on day 77, an ear bleed from each rabbit was taken for analysis. Finally, 91 days after the initial injections, both rabbits were bled-out via cardiac puncture (approximately 70 mL from each rabbit). Collected blood was allowed to clot for at least 1 h (22°C), and the antiserum was decanted and stored in 1.5 mL sealed containers at -20°C. At the time of the bleed-out, the antisera of both rabbits were of equal strength and performance. Because strong titers were obtained with both rabbits, only the antiserum from one rabbit was used in all EIA experiments.

Competitive Indirect EIA Procedure

A 96-well microtiter plate was coated with 200 µL/well of a 10 part per billion (ppb) solution (w/v) of an azo- or hemisuccinate-linked competitor conjugate in PBS. The plate was protected from evaporation with an acetate plate sealer and left overnight at 4°C. The next day the coating solution was shaken from the wells and the plate coated with a 1% BSA in PBS solution. The plate was sealed with a plate sealer and left to incubate for 1 h

at room temperature. After 1 h, the 1% BSA in PBS solution was shaken from the plate and the wells washed 3 times with PBST, shaking and blotting the PBST from the wells onto paper towels between each washing. Aqueous sodium SMR dilutions (10 µg/mL - 1 fg/mL) were added (100 µL/well) as a source of free hapten in solution. To some of the wells, 100 µL of water was added as a control. Antiserum diluted 500,000 times with 0.05% BSA in PBST was added (100 µL/well) and the plate sealed and incubated for 2 h. Following the incubation, the solution was shaken from the wells and the plate was washed and blotted 3 times as previously described. Goat anti-rabbit peroxidase-conjugated antibodies diluted 3000 times with PBST were added to all the wells (200 µL/well), and the plate incubated with a plate sealer for a further 2 h at room temperature. After washing the plate with PBST as before, 200 µL of a substrate solution (TMB (0.1 mg/mL) and urea peroxide (1.0 mg/mL) dissolved in 0.1 M citrate buffer, pH 4.0) was added to each well. The enzyme-substrate reaction was stopped after 7 min (azo-linked conjugates) or 5 min (hemisuccinate-linked conjugates) with the addition of 50 µL/well of 2.0 M sulfuric acid, and the absorbance read at 450 nm.

EIA Data Analysis

The I_{50} value (ppb of SMR) was used as a comparison point for each of the conjugates. The I_{50} value was the inflection point of a best-fit, four parameter curve (equation (5)) calculated by SOFTmax (version 2.01) software (Molecular Devices Corp., Menlo Park, CA).

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d \qquad (5)$$

Where: a and d are the upper and lower asymptotes
b is the slope of the linear portion of the curve
c is the inflection point (middle) of the curve (I_{50})

To prepare the data for the SOFTmax program, a blank value for the plate was subtracted from the measured absorbance value for each well. The blank value for the plate was measured by taking the average absorbance from at least 12 wells on the plate, where a 1% BSA in PBS sclution was used for coating the wells instead of either an azo- or

hemisuccinate-linked competitor conjugate solution. All other additions of solutions to the plate were the same for the 1% BSA in PBS coated wells as that used for the competitor conjugate coated wells. Once the blank value had been removed from the absorbance value of each well, a value for B (absorbance of a well containing SMR) divided by B_0 (absorbance of a well without SMR) could be calculated for each well. The B/B_0 values and their corresponding concentration of SMR (ppb) were then processed by the SOFTmax software. For additional information, refer to Appendix I for samples of competitive indirect EIA data, and a sigmoidal curve and its parameters generated from competitive indirect EIA data, using SOFTmax software.

RESULTS AND DISCUSSION

Research has shown that the method chosen for hapten linkage is important for EIA performance (Sheth and Sporns, 1991; Wie and Hammock, 1984; Vallejo *et al.*, 1982). When an immunogen is injected into rabbits, antibodies are created which have affinity for the carrier protein, the hapten, and the region by which both are linked (the linking arm). Under properly selected conditions in the competitive indirect EIA, hapten-specific antibodies in the antiserum can be preferentially utilized, allowing for better competition and a decreased background. This can be accomplished through the use of different carrier proteins for the immunogen and the conjugate used to bind the hapten to the microtiter plate well (competitor conjugate), and by changing the hapten-protein linking methods for the competitor conjugates and the immunogen.

Improved EIA detection capabilities have also been found when the amount of hapten bound to the well of the microtiter plate is limited (Ekins and Chu, 1991; Manning, 1991; Porstmann and Kiessig, 1992). Theoretically, by limiting the amount of plate-bound hapten, there is a requirement for less hapten in solution to compete for the hapten-specific antibody in the antiserum. A range of 3 to 25 molecules of hapten per molecule of protein has been suggested for a successful EIA of small molecules (Roe, 1991).

In this study we examined not only the effect of a change in the hapten (SMR) to protein carrier (BSA) linking method, but also the effect that a change in the number of hapten molecules per mole of protein carrier (hapten-protein molar ratio) has on the performance of the EIA competitor conjugates. However, any relationship between hapten-protein molar ratios and EIA detection capabilities can only be established if the hapten-protein molar ratios are accurately known.

Determination of Hapten-Protein Molar Ratios for Azo-linked Conjugates

For the azo-linked conjugates, the following methodologies were explored to determine a reliable technique for measuring the number of hapten molecules bound to the protein carriers.

Microanalysis Method. SMR contains 49.98% carbon, 4.58% hydrogen, 21.20% nitrogen, 12.13% sulfur, and 12.11% oxygen. Microanalysis of the conjugates led to unsatisfactory results since differences in the carbon, hydrogen, nitrogen, and sulfur percentages of the SMR-BSA conjugates, when compared with the analysis results of BSA alone, were less than or equal to the margin of error (± 0.3%) expected from this analysis.

Immunological Method. The use of immunological methods for determining hapten-protein molar ratios has been described by Morgan *et al.* (1983) and Wie and Hammock (1984). Generally, the protocol used in this form of determination, and which was employed in our study, involves the use of the competitor conjugate as a source of free and bound SMR. After the competitor conjugate solution was used to coat the wells of the microtiter plates (solid phase hapten), it was further diluted and used as a source of liquid phase hapten in the antibody-binding competition stage of the EIA. Through use of different combinations of azo- and hemisuccinate-linked competitor conjugates for coating, and as a source of liquid phase hapten (azo conjugate for coating and in solution, azo conjugate for coating and hemisuccinate conjugate in solution, hemisuccinate conjugate for coating and in solution, hemisuccinate conjugate for coating and azo conjugate in solution), sigmoidal curves for conjugate competition were obtained. The resultant sigmoidal curves were compared to standard curves prepared from competitive indirect EIAs (where the same competitor conjugate had been used for plate coating) using SMR as the source of liquid phase hapten for competition. Through these competition experiments, it was concluded that the antiserum antibodies had a considerably greater affinity for protein-bound hapten than free hapten. Therefore, reasonable values for the hapten-protein molar ratios of the competitor conjugates could not be determined using this methodology.

^{14}CSMT-BSA Standard Curve Method. The ^{14}CSMT-BSA standard curve approach involved the determination of hapten-protein molar ratios through the preparation of a radioactive protein conjugate from commercially available ^{14}C labeled SMT. Radioactive SMT was used because radioactively-labeled SMR was not available. It was proposed that the absorbance spectrum of an azo-linked conjugate prepared with SMR, could safely be equated to that prepared with SMT for the following reasons. SMR is very similar to SMT in structure and absorbance spectrum. The diazo reaction used in the production of the sulfonamide chromophore utilizes the amino group at the opposite end of the molecule from where the difference of one methyl group distinguishes SMR from SMT (Figure 2.1). In addition, it has been reported that the molar absorptivities of the chromogenic products of all diazotized sulfonamides are approximately equal (Horwitz, 1981). Therefore, a standard curve was prepared relating the A_{430nm} of the ^{14}CSMT-BSA conjugates to the molar ratios of hapten-to-protein for these conjugates determined directly by radioactive decay. From this standard curve, knowing the A_{430nm}, hapten-protein molar ratios for the azo-linked competitor conjugates were calculated (Table 2.2). This method was determined to be the best for estimating the degree of hapten linkage, because hapten number was directly related to absorbance of the conjugate solutions without the necessity of determining the precise source of the chromophore.

Amino Acid Analysis Method. Amino acid analyses were performed on only SMR-BSA(L) and SMR-BSA(M), as SMR-BSA(H) had not yet been synthesized. The hemisuccinate conjugates were not suitable for analysis since their hemisuccinate linking arm would be susceptible to hydrolysis during sample preparation. The theory behind these analyses was that the conditions of hydrolysis required for sample preparation would not affect the azo linkage between SMR and the individual amino acids. This would result in a modified amino acid residue of either tyrosine or histidine which would elute from the cation exchange column with different retention times than the unmodified amino acids. Equation (4) was used to quantitate the degree of amino acid modification through comparison of the amino acid composition found for BSA, and the compositions found for the two azo-linked competitor conjugates (SMR-BSA(L) and SMR-BSA(M)). Individual calculations were performed using either the concentration of glycine or arginine as an internal standard. Glycine and arginine were chosen because their structures were not affected by the conditions of the acid hydrolysis. Also, these two amino acids were eluted from the column before (glycine) and after (arginine) the tyrosine and histidine residues. This made them suitable amino acids to compensate for the varying concentrations of protein hydrolysate applied to the column for analysis. The values of N for tyrosine and histidine, 20 and 17, respectively, represent the number of tyrosine or histidine residues of the 583 amino acid residues which comprise a BSA molecule (Hirayama *et al.*, 1991). Examination of the results (Table 2.2) revealed that the primary amino acid involved in our azo-linked proteins was histidine, and that there was minimal involvement of the tyrosine residues in the light and medium loaded azo conjugates examined. This preferential linkage to histidine in our conjugates was possibly due to the method of conjugate synthesis where the conditions were not very basic. Assisting the reaction of the diazonium ion with tyrosine is the deprotonation of the hydroxyl group of tyrosine (pKa of 10.07; *The Merck Index*, 1989). In comparison, the pKa of the ionizable nitrogen on histidine is 5.97 (*The Merck Index*, 1989). Because our reaction of the sulfonamide diazonium ion with BSA was carried out at a pH greater than eight, the majority of the histidine residues, and not the tyrosine residues, would be ionized. The slightly lower values for the hapten-protein molar ratios obtained with amino acid analysis, compared with those reported by the ^{14}CSMT-BSA method, may have resulted from the inability of amino acid analysis to account for the contribution of azo-linked tryptophan residues. Tryptophan residues account for two of the 583 amino acids in BSA (Hirayama *et al.*, 1991), and were considered by Howard and Wild (1957) to be possible sites for azo attachment in proteins. Tryptophan, however, is destroyed by the acidic conditions required to prepare the conjugates for amino acid

analysis, and therefore the extent of involvement of this amino acid in the azo reaction is unknown.

NCAT/NAH Standard Curve Method. Spectrophotometric analyses were attempted with the azo-linked conjugates knowing that the production of colored compounds primarily involved the amino acids tyrosine and histidine (Howard and Wild, 1957). This spectrophotometric method for determining hapten to carrier protein ratios was reported by Fenton and Singer (1971) and was subsequently used by others (Dargar *et al.*, 1991; McAdam *et al.*, 1992) for similar azo reactions but with different haptens. Hapten-protein molar ratios for each of the competitor conjugates were derived from standard curves prepared using NCAT and NAH. NCAT and NAH are the protected amino acid analogs of tyrosine and histidine, respectively. These analogs were used because they had the reactive amino group blocked, preventing competition for the diazonium ion by the basic amino group (Tabachnick and Sobotka, 1959). In preparing the NCAT and NAH standard curves, the reaction conditions employed closely mimicked those used to produce the azo-linked conjugates. However, this method for the determination of SMR to protein ratios did not correlate well with results obtained using two earlier methods, ^{14}CSMT-BSA standard curve and amino acid analysis (Table 2.2).

To examine why the NCAT/NAH standard curve method did not provide the expected values, a closer examination of the individual NCAT and NAH curves was undertaken. Although suitable coefficients of determination ($r^2 \geq 0.98$) were obtained for the reaction of NCAT with SMR, the slope of the NCAT standard curve (1082 absorbance units/M), was approximately 10 times less than the extinction coefficient of 10,400 reported from a similar diazo reaction by Traylor and Singer (1967). This indicated that our reaction conditions may not have been suitable to produce the maximum amount of product from the initial quantities of the NCAT and SMR reactants. This would result in an overestimation of the contribution of azo-linked tyrosine residues in the conjugates by equations (1) and (2). Also, it was determined from amino acid analyses that only azo-linked histidine residues were found when a limited amount of diazonium ion was involved in the reaction. An attempt was then made to discover whether an accurate estimation of the hapten-protein molar ratio could be determined spectrophotometrically, if only the NAH standard curve was used. The slope of the NAH standard curve relating the A_{436nm} and the molar concentration of SMR was 22,460 absorbance units/M ($r^2 = 1.0$). This was very close in value to the extinction coefficient of 22,600 reported by Traylor and Singer (1967) using a similar NAH-diazo reaction. Table 2.2 gives the values obtained by using the NAH standard curve at 436 nm. However, when compared with the ^{14}CSMT-BSA

standard curve, the results using the NAH standard curve at 436 nm still underestimated the hapten-protein molar ratio for each competitor conjugate.

With direct measurements of radioactive decay as the best estimation of hapten-protein molar ratios, a radioactively-labeled SMT conjugate (^{14}CSMT-BSA(2)), synthesized independently of those used to create the ^{14}CSMT-BSA standard curve, was used in a final evaluation of the spectrophotometric methodologies. The results obtained for the hapten-protein molar ratios, using this conjugate, are reported in Table 2.4. Amino acid analysis was not performed on this conjugate, because the containment of radioactivity could not be satisfactorily guaranteed. The trend in the results observed for the azo-linked competitor conjugates was similar to that found for the ^{14}CSMT-BSA(2) conjugate. The NCAT/NAH standard curves overestimated while the NAH standard curve (436 nm) underestimated the hapten-protein molar ratio for ^{14}CSMT-BSA(2). Only the ^{14}CSMT-BSA standard curve was able to provide an estimation of the hapten-protein molar ratio that was reasonably close to the value calculated from radioactive decay measurements of the ^{14}CSMT-BSA(2) conjugate. It was concluded that, for our hapten, the validity of comparing the absorbance of a chromophore derived from a single individual amino acid derivative with that of the same amino acid while part of a larger protein structure is questionable, and may lead to erroneous results.

Determination of Hapten-Protein Molar Ratios for Hemisuccinate-linked Competitor Conjugates

Prior to the success of the water soluble carbodiimide method (using EDC) for the synthesis of the hemisuccinate-linked competitor conjugates, experiments were performed using the *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (NHS/DCC) reaction for active ester formation, and succinylsulfamerazine linkage to BSA. However, in the attempts that were made using the NHS/DCC method, only minute quantities, if any, of hemisuccinate-linked conjugate could be produced. Observed precipitation of the SMR active ester on addition to the BSA solution, and poor yields of conjugate, were probably the result of formation of a cyclic succinylsulfamerazine (reaction (5), Figure 2.1). The occurrence of a cyclic form during the formation of hemisuccinate-linked conjugates has been described by Tatake *et al.* (1991) and Roseman *et al.* (1992).

Sulfanilic Acid Standard Curve. The sulfanilic acid standard curve was used to determine the values for the hapten-protein molar ratios of the hemisuccinate-linked competitor conjugates. This was accomplished by first digesting each of the conjugates in refluxing hydrochloric acid. The digestion served to decompose the hemisuccinate-linked conjugates, producing sulfanilic acid from the once-bound hapten. The aromatic amino

Table 2.4. Hapten-Protein Molar Ratio Determined for the ^{14}CSMT-BSA(2) Conjugate

Method of Analysis	^{14}CSMT-BSA(2) Conjugate (moles hapten/mole protein)
Radioactive Decay	6.0
NCAT/NAH standard curve ($M_{SMR\text{-}NCAT}$[a])	13.7
($M_{SMR\text{-}NAH}$[b])	1.7
(total)	15.4
NAH standard curve (A_{436nm})	2.1
^{14}CSMT-BSA standard curve	5.2

[a] values for moles hapten/mole protein were calculated using the molar concentration of ^{14}CSMT/SMT derived from equation (1), and the known concentration of protein used for absorbance measurements.

[b] values for moles hapten/mole protein were calculated using the molar concentration of ^{14}CSMT/SMT derived from equation (2), and the known concentration of protein used for absorbance measurements.

group of sulfanilic acid would then be free to react under the conditions of the modified form of the B-M reaction to produce a quantifiable color. The length of digestion was optimized until an error of less than 2 μg/mL of the actual concentration was achieved with sulfanilic acid, SMR, and SMR-SA standards.

Comparison of Azo- and Hemisuccinate-linked Competitor Conjugates in a Competitive Indirect EIA

Assay Optimization. The six competitor conjugates synthesized for this study were categorized as light, medium, or heavy depending on their hapten-protein molar ratios. The intra-comparison of the azo- and hemisuccinate-linked competitor conjugates was performed on conjugates synthesized with the same hapten-protein linking method. The inter-comparison of the azo versus the hemisuccinate-linked competitor conjugates was permissible because the light, medium and heavy loadings of hapten-protein for each linking method were approximately equal. To allow for the least bias in evaluation of the performance of all six competitor conjugates, a common set of conditions was established for the competitive indirect EIA through the use of a checkerboard EIA. A 10 ppb dilution of competitor conjugate, and a 500,000 times dilution of the antiserum, were found to be the best compromise of conditions for the intra- and inter-conjugate comparisons.

Competitive Indirect EIA Results. The detection capabilities of each of the competitor conjugates were compared using the I_{50} values (Table 2.5). The values indicated that the azo-linked competitor conjugates were distinctly superior to the hemisuccinate-linked competitor conjugates. From within the two groups of conjugates, it was the lightly loaded conjugates, SMR-BSA(L) and SMR-SA-BSA(L), that outperformed their more heavily loaded counterparts (refer to Appendix I for competitive indirect EIA data, and SOFTmax processed competitive indirect EIA data, for SMR-BSA(L) and SMR-SA-BSA(L)). This was consistent with the theory that the least amount of hapten bound to the plate would provide the best competition for hapten in solution, for the limited number of antibody binding sites. Unexpectedly, the results also demonstrated that the azo-linked competitor conjugates were generally superior to the hemisuccinate competitor conjugates. Since an azo-linked conjugate (SMR-LPH) was used for immunization, it was expected that the linking region would cause a shift in antibody binding preference towards the azo-linked competitor conjugates, and away from hapten in solution. This should have led to higher detection limits, or greater I_{50} values, for the azo-linked competitor conjugates. It was also thought that by using a hemisuccinate linking arm for the competitor conjugates, problems with undesirable antibody binding associated to the linking region would be eliminated. This should have decreased the antibody bias for the plate-bound hapten of the

Table 2.5. I_{50} Values for Azo- and Hemisuccinate-linked Competitor Conjugates

Competitor Conjugates	I_{50} Values for SMR
Azo-linked:	
SMR-BSA(L)	3.4 (12)[a]
SMR-BSA(M)	7.7 (27)
SMR-BSA(H)	7.0 (25)
Hemisuccinate-linked:	
SMR-SA-BSA(L)	31 (108)
SMR-SA-BSA(M)	53 (185)
SMR-SA-BSA(H)	66 (231)

[a] ppb value followed by equivalent nM value in parenthesis

hemisuccinate-linked competitor conjugates, when compared to that of hapten in solution, improving competition and lowering detection limits (or lowering I_{50} values) for these conjugates.

Therefore, this study revealed that the azo-linked competitor conjugates outperformed their hemisuccinate counterparts, contrary to theoretical expectations. However, minimizing hapten-protein molar ratio did provide the expected result of an improved EIA detection limit.

The azo-linked conjugate (SMR-BSA(L)) and hemisuccinate-linked conjugate (SMR-SA-BSA(L)) were determined to be the competitor conjugates with the lowest levels of detection for SMR. Chapter 3 details the next phase of evaluation where SMR-BSA(L) and SMR-SA-BSA(L) were used for the practical application of detecting SMR in contaminated milk from sulfonamide treated cows.

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Chapter: 3

AN ENZYME IMMUNOASSAY FOR SULFAMERAZINE. 2. Evaluation of Assay Performance with Milk Samples*

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INTRODUCTION

Antimicrobial agents are important agricultural tools that animal producers use to keep livestock healthy and their businesses economically viable. Unfortunately, the therapeutic treatment of livestock with a group of antimicrobial agents known as sulfonamides has become a recent concern as sulfonamide residues have been discovered in cows' milk destined for human consumption (Brady and Katz, 1988; Charm *et al.*, 1988; Collins-Thompson *et al.*, 1988; Larocque *et al.*, 1990). Sulfonamides endanger human health through allergic reactions (Huber, 1986), and their suspected carcinogenicity (Littlefield *et al.*, 1990). They also pose an economic hazard through the inhibition of the growth of dairy starter cultures (Schiffmann *et al.*, 1992).

Many commercial screening tests for sulfonamides have been developed for milk. Some tests, such as the Charm II microbial receptor assay and microbial inhibition tests, are able to detect all or the majority of sulfonamides. However, Cullor (1992) and Tyler *et al.* (1992) have suggested that the immunological response of cows could result in the reporting of false positives for many of these microbial assays, and the only test free of this interference was a competitive enzyme immunoassay (Tyler *et al.*, 1992). Enzyme immunoassays (EIA) are sensitive and reliable analytical techniques, and tests such as the LacTek (Idetex Inc., Sunnyvale, CA) are capable of a 10 part per billion (ppb) detection limit for sulfamethazine (SMT). However, commercial EIAs detect only individual sulfonamides, and most detect only SMT. The United States Food and Drug Administration (FDA) has been cited as considering 10 ppb as a safe level for all sulfonamides (Zomer *et al.*, 1992). Therefore, there is a need for EIAs that detect sulfonamides other than SMT at very low levels. An FDA report (National Survey of Shelf Milk for Sulfonamides and Tetracyclines, April 3, 1990) has indicated that one of the major sulfonamide contaminants in milk is sulfamerazine. While sulfamerazine (SMR) is very similar in chemical structure to SMT, EIAs are so specific that a SMT EIA would be considerably less sensitive for SMR. To our knowledge, there have not been any screening tests developed specifically for SMR in milk.

In chapter 2, EIAs were prepared for the detection of SMR. That study examined the effect that the choice of hapten-protein linking method and hapten-protein molar ratio had on the performance of a competitor conjugate in an EIA. In this study, two of the SMR EIAs developed in chapter 2 (utilizing a competitor conjugate from each hapten-protein linking method) were used to detect SMR in milk from cows treated with the aqueous sulfonamide supplement, 3-Sulvit. In addition to the SMR EIAs, two commercial tests (the Charm II assay and the CITE Sulfa Trio test) were used to analyze the

sulfonamide content of the milk from the 3-Sulvit treated cows. The Charm II assay and the CITE Sulfa Trio test are capable of detecting sulfonamides in milk at levels of at least 10 ppb, and were useful for evaluating the performance of the SMR EIAs.

EXPERIMENTAL

Instrumentation

The EIA analyses were performed in 96-well, Immulon 2, flat-bottomed microtiter plates from Dynatech Laboratories Inc. (Chantilly, VA) with Linbro nonsterile acetate plate sealers manufactured by ICN Biomedicals Inc. (Costa Mesa, CA) to prevent evaporation. Absorbance measurements were made using a Model EL 309 microplate reader (Bio-Tek Instruments, Inc., Burlington, VT).

Reagents

Sodium sulfamerazine (SMR), sodium sulfamethazine (SMT), sodium sulfathiazole (STZ), sodium sulfadimethoxine (SDX), bovine serum albumin (BSA), 3,3', 5,5'-tetramethylbenzidine dihydrochloride (TMB), thimerosal, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Urea peroxide, and goat anti-rabbit peroxidase-conjugated antibodies were obtained from Calbiochem Co. (San Diego, CA). 3-Sulvit was produced by Sanofi Santé Animale (Victoriaville, PQ).

Homogenized milk obtained in 1 liter containers (having 3.25% milk fat as reported on the container) was produced by the Northern Alberta Dairy Pool Ltd.(Edmonton, AB).

All solutions were prepared using water purified by a Millipore Milli-Q system (Millipore Corp., Millford, MA) and all other chemicals used were of reagent grade or better.

Preparation of Sulfonamide-treated Bovine Milk

A herd of 55 holstein dairy cows managed by the University of Alberta Dairy Unit was the source of raw milk for this study. The herd was milked twice daily using a single 8 herring bone milking parlor and the milk was stored in a refrigerated bulk tank. At the time of this study, the cattle were not undergoing any antimicrobial treatment, and the bulk tank was the source of sulfonamide-free raw milk. Three cows with identification numbers 8421, 8881, and 9020 were chosen for treatment with 3-Sulvit (composition shown in Table 3.1) based on their stage of lactation and the number of days into their milking cycle. Each of the cows had been milked for 150-200 days, well into their milking cycle, which eliminated any potential problems that might be associated with colostrum milk. Data on the three cows are given in Table 3.2. Milk samples (100-200 mL) were taken from the three cows prior to the administration of 3-Sulvit (day 1/am), which provided for the background response level for each animal.

Table 3.1. Composition of 3-Sulvit[a]

Active Ingredient	Amount per package	% Weight based on package weight (450 g)
Sulfamethazine, sodium	170.50 g	37.9
Sulfamerazine, sodium	67.40 g	15.0
Sulfathiazole, sodium	136.30 g	30.3
Vitamin A	240,000.00 I.U.	
Vitamin D_3	80,000.00 I.U.	
Riboflavin	200.00 mg	
Niacinamide	3.00 g	
d-Pantothenic acid	700.00 mg	
Vitamin B_{12}	2.30 mg	
Magnesium sulfate	200.00 mg	
Sodium acetate	2.80 g	
Sodium chloride	3.15 g	
Calcium chloride	250.00 mg	
Potassium chloride	250.00 mg	

[a] composition of 3-Sulvit as defined by package label

Table 3.2. Statistical Data of Treated Cows

Cow Identification Number	Approximate Weight (kg)	Stage of Lactation	3-Sulvit Administered (g)	Average Milking in kg/day[a] (day 1/am-7/am)
8421	630	7	60.8	25.3
8881	580	2	60.3	17.6
9020	525	1	60.1	25.2

[a] daily milkings varied by no more than $\pm$ 3 kg

3-Sulvit treatment was administered to each cow (approximately 5 h after the day 1/am milking) by means of a stomach drench. Stomach drenching involved pumping, from a plastic container, an aqueous solution (approximately two liters) of the 3-Sulvit through a hose directly into the cow's stomach. This was immediately followed by pumping 1 to 2 L of water to wash any remaining 3-Sulvit into the stomach from the plastic container and hose.

The level of 3-Sulvit chosen for treatment of the cows was based on package recommendations with some modification. The package suggested dissolving one pouch (450 g) in 1350 L of drinking water, when administered to cattle. With each holstein cow drinking, on average, 120 L of water a day (estimated by the University of Alberta Dairy Unit manager), a cow would then be expected to ingest 40 g of 3-Sulvit/day. For this study, a treatment of 60 g/stomach drench of 3-Sulvit was chosen (1.5 times the calculated daily intake) to ensure that an adequate dose of the sulfonamides was excreted by the cow into her milk. With sulfonamides composing approximately 83% of the package weight of 3-Sulvit, the oral dose that each cow received would be (on average) 87 mg of sulfonamides/kg body weight. Other authors who have orally treated cows with sulfonamides have used from 26.4-220 mg of sulfonamide/kg body weight (Paulson *et al.*, 1992a). There have also been reports of dairy cattle tolerating single intravenous doses of approximately 160 mg/kg body weight of SMR, SMT and sulfathiazole (STZ) (Rehm *et al.*, 1986).

Treated cows were tagged and milked separately from the rest of the herd, and 100-200 mL samples of their milk were collected from the total morning (am) and evening (pm) milkings, and immediately frozen for later analysis. There were approximately nine hours between the am and pm milkings for each cow. After sampling, the remaining sulfonamide contaminated milk was discarded. This process was continued until the treated cow's milk contained less than 10 ppb of residual sulfonamide as determined by Charm II analyses. When the cow's milk contained less than 10 ppb of residual sulfonamide, it was determined to be safe to put the cow back on-line with the rest of the herd. The treated cows demonstrated no apparent ill after effects from either the stomach drenching procedure or the 3-Sulvit treatment.

Testing of the pre-3-Sulvit and 3-Sulvit treated cows' milk samples, for each cow, were run on three consecutive days. The morning of testing, the milk samples from one cow were thawed, and all three test formats (Charm II, SMR EIA, and CITE Sulfa Trio) were performed on the milk samples that day, while keeping the samples in an ice bath.

Preparation of Sulfonamide-spiked Homogenized Milk Samples

Homogenized milk (produced by the Northern Alberta Dairy Pool Ltd. but purchased from a retail store) was used for the preparation of a SMR standard curve and for cross-reactivity studies with SMT, STZ and sulfadimethoxine (SDX). Each of the sulfonamides was first dissolved in water, then a 10-fold dilution of the aqueous solution was made with milk. The sulfonamide/milk solutions were then used to make further 10 fold serial milk dilutions of each of the sulfonamides to be used in test evaluations. For the standard curves, milk dilutions of sulfonamide ranged from 1 µg/mL to 0.01 fg/mL, and for the cross-reactivity studies, milk dilutions of sulfonamide ranged from 10 µg/mL to 0.01 ng/mL.

Charm II Microbial Receptor Assay Procedure

The Charm II analyses were performed at the Northern Alberta Dairy Pool processing plant using equipment and reagents purchased from Charm Sciences Inc. (Malden, MA). The procedure used in analysing the milk samples was the "antimicrobial sulfa drug sequential assay", as outlined in the operator's manual for the Charm II assay. The Charm II assay, by the manufacturer's claims, should detect all three sulfonamides (SMT, SMR, and STZ) found in 3-Sulvit. In the sequential assay, sulfonamides present in the milk sample were given the first opportunity to bind to the receptor molecule. Then a ^{3}H labeled SMT drug tracer was added to the milk sample which bound to any remaining receptor sites. The receptor/sulfonamide complex was centrifuged, any unbound radioactivity and free sulfonamides were decanted and discarded, and the radioactivity of the remaining complex was measured after the addition of scintillation fluid. The amount of radioactivity in counts per minute (cpm) is inversely proportional to the amount of sulfonamide in the milk sample. By performing the sequential assay on a "positive control" (a reconstituted milk powder containing 10 ppb of SMT) supplied by Charm Sciences, a relationship between the radioactivity (cpm) measured in the assay and a 10 ppb SMT milk solution was established. Through numerous trials, a 10 ppb sulfonamide control point was set when the Charm II analyzer gave a fixed display of 1100 cpm. Therefore any milk samples with counts less than 1100 cpm contained in excess of 10 ppb of sulfonamide.

CITE Sulfa Trio Test Kit Procedure

The CITE Sulfa Trio test kits were obtained from IDEXX Laboratories, Inc. (Westbrook, ME). The CITE Sulfa Trio is a membrane-immobilized, antibody-based test, comprised of a control, and three test spots specific for the sulfonamides SMT, STZ and SDX. As described in the CITE Sulfa Trio test kit manual, the test utilizes competitive

binding for antibody binding sites established between residual sulfonamide in the milk, and an enzyme-labeled sulfonamide standard solution added to the milk sample. After washing, and upon addition of substrate, a blue precipitate, proportional to the amount of bound enzyme-labeled sulfonamide, is formed by the enzyme reaction. Therefore, the formation of blue precipitate is inversely proportional to the amount of sulfonamide present in the original sample. Visual inspection of precipitate in each of the sulfonamide designated regions of the test cups was used to determine whether the milk samples contained greater or less than 10 ppb of SMT, STZ or SDX. From analyses performed by SMR EIAs and Charm II analyses, the range of milk samples which could provide information using the CITE Sulfa Trio was narrowed to 4 or 5 samples. Milk was tested until a sample was found which could be visually identified by the test as containing approximately 10 ppb of SMT or STZ.

Phosphate-Buffered Saline (PBS and PBST) Solutions

The PBS solution was prepared by dissolving sodium chloride (9.0 g), disodium hydrogen phosphate (1.108 g), potassium dihydrogen phosphate (0.3 g) and thimerosal (0.1 g) in 1 liter of water. To prepare PBST, 0.5 g (0.05%) of Tween 20 was added to the PBS solution prior to dilution to volume with water. The pH of the PBS or PBST was adjusted to 7.3 with sodium hydroxide or hydrochloric acid as needed.

Preparation of Polyclonal Antibodies and Competitor Conjugates

A description of the preparation of the polyclonal antibodies and the azo- and hemisuccinate-linked competitor conjugates that were used for milk analysis was given in chapter 2. Briefly, polyclonal antibodies were derived from the serum of a Flemish Giant x Dutch Lop Ear rabbit, injected with an azo-linked sulfamerazine-*Limulus polyphemus* hemolymph conjugate. When tests showed that the serum had sufficient titer for use in the EIA experiments, the rabbit was bled out and the serum stored at -20°C.

The lightly loaded, sulfamerazine-bovine serum albumin azo-linked competitor conjugate, (SMR-BSA(L)), was prepared using an azo linkage between the N^4 terminal amine group of SMR, and BSA. This resulted in a hapten-protein molar ratio of 4.3 moles of SMR per mole of BSA. The preparation of the lightly loaded, sulfamerazine-bovine serum albumin hemisuccinate-linked conjugate, (SMR-SA-BSA(L)) was accomplished with the use of a succinate linkage between SMR and BSA. This resulted in a hapten-protein molar ratio for this conjugate equal to 6.4.

Competitive Indirect EIA Procedure

Each well of a 96 well microtiter plate was coated with 200 µL of a 10 and 20 ng/mL PBS solution of the hemisuccinate and azo-linked competitor conjugate, respectively. The plate was protected from evaporation with an acetate plate sealer and left overnight at 4°C. The next day the coating solution was shaken from the wells and the plate coated with a 1% BSA in PBS solution. The plate was then sealed with a plate sealer and left to incubate for 1 h at room temperature. After 1 h, the 1% BSA in PBS solution was shaken from the plate and the wells washed 3 times with PBST, shaking and forcibly blotting the PBST from the wells between each washing. For the SMR standard curve, sulfonamide cross-reactivity studies, and 3-Sulvit medicated cows' milk samples, 100 µL/well of each of the serial dilutions of sulfonamide in milk was added to the wells as a source of free hapten. To some of the wells, 100 µL of milk (homogenized with no sulfonamide added or premedicated cows' milk samples) was added as a control. Serum diluted 500,000 times (azo-linked competitor conjugate) and 1,000,000 times (hemisuccinate-linked competitor conjugate) with 0.05% BSA in PBST was added (100 µL/well) and the plate sealed and incubated for 2 h. Following incubation, the solution was shaken from the wells and the plate was washed and blotted 4 times as previously described. Goat anti-rabbit peroxidase-conjugated antibodies diluted 3000 times with PBST were added to all of the wells (200 µL/well), and the plate was incubated with plate sealers for a further 2 h at room temperature. After washing the plate with PBST as before, 200 µL of a substrate solution (TMB (0.1 mg/mL) and urea peroxide (1.0 mg/mL) dissolved in 0.1 M citrate buffer, pH 4.0) was added to each well. The enzyme-substrate reaction was stopped with the addition of 50 µL/well of 2 M sulfuric acid and the absorbance at 450 nm was read.

EIA Data Analysis

The data from the competitive indirect EIA experiments, with both homogenized and sulfonamide contaminated raw milk, were processed using SOFTmax (version 2.01) software (Molecular Devices Corp., Menlo Park, CA) into a best-fit, four parameter curve (equation (5), chapter 2). The point of inflection of a sigmoidal curve which has been designated as the I_{50} value (nM), was chosen as the most reliable point for determining the degree of sulfonamide cross-reactivity with the polyclonal antibodies. To prepare the data for the SOFTmax program, a blank value for the plate was subtracted from the measured absorbance value for each well. The blank value for the plate was measured by taking the average absorbance from at least 12 wells on the plate, where a 1% BSA in PBS coating was used instead of either the azo or hemisuccinate-linked competitor conjugates. All other

additions of solutions to the plate remained the same for the 1% BSA in PBS coated wells as for the competitor conjugate coated wells. Once the blank value had been deducted from the absorbance value of each well, a value for B (absorbance of a well containing sulfonamide contaminated milk) divided by B_0 (absorbance of a well with milk but no sulfonamide) could be calculated for each well, and the B/B_0 values then processed by the SOFTmax software. For the standard curve and cross-reactivity studies the values of B_0 were obtained using homogenized milk (no added sulfonamide) and for the 3-Sulvit treated cows' milk samples, B_0 was obtained from the day 1/am milk (milk sample taken prior to 3-Sulvit treatment) of each cow.

Data Processing for SMR EIA Standard Curves, and Sulfonamide-contaminated Raw Milk Samples

Sigmoidal standard curves were produced using SMR-spiked homogenized milk and the SOFTmax software, for both the SMR-BSA(L) and SMR-SA-BSA(L) competitor conjugates (refer to Appendix II for competitive indirect EIA data). The equations of the sigmoidal curves for each competitor conjugate were used to convert the absorbance values (B/B_0) from the 3-Sulvit treated cow's milk samples into numerical values expressed in ppb (refer to Appendix II for parameters used in the best fit, 4-parameter equation). All values less than 100 ppb were reported for the milk samples. A 20-80% reduction from the upper asymptote of each of the sigmoidal standard curves represented the linear region of the curves, and ppb values derived from this region were considered the most accurate. For the sigmoidal standard curve produced using SMR-BSA(L) as the competitor conjugate, the concentration of SMR within the linear region was from 0.15-5.7 ppb. For the sigmoidal standard curve produced using SMR-SA-BSA(L) as the competitor conjugate, the concentration of SMR within the linear region was from 0.68-16.6 ppb. Triplicate analyses were performed on each of the SMR dilutions used to prepare the standard curves and the coefficient of variation of the absorbance values (V_A) were calculated for the linear region of the curves. For the SMR-BSA(L) sigmoidal standard curve, the V_A average was 5.4%, and for the SMR-SA-BSA(L) sigmoidal standard curve, 12.4%. Analyses of the cows' milk samples were also performed in triplicate. For all the sulfonamide contaminated cows' milk samples, the average V_A for numerical values obtained using the SMR-BSA(L) competitor conjugate was 6.8%. The average V_A numerical values obtained for the cows' milk samples using the SMR-SA-BSA(L) competitor conjugate was 5.6%.

Sulfonamide Contaminated Milk Samples

SMR-spiked Milk Samples. Preparation of the SMR standard curve was attempted using sulfonamide-free raw milk obtained from the bulk milk tank at the University of Alberta Dairy Unit. When 10-fold serial dilutions of SMR in the raw milk were tested in the EIAs, it was found that there were substantial fluctuations in the absorbance values of the highest dilutions. These fluctuations made it difficult to obtain a consistent standard curve and especially difficult to obtain an accurate I_{50} value from the sigmoidal curve. It was believed that the fluctuations were the result of the difficulty in spiking raw milk, which is a two phase system (predominantly fat and predominantly aqueous phases), with SMR at very low levels. The fluctuations in absorbance values of highly diluted SMR samples did not occur with homogenized milk. Although the fat content in homogenized milk is approximately the same as that of the raw milk tested, the homogenized milk, unlike the raw milk, did not separate out during the 2 h incubation step. Even though all of the samples were mixed vigorously between dilutions, the hydrophobic SMR (Long *et al.*, 1990) was likely in greater concentrations in the predominantly fat layer of the separated raw milk. Minute changes in the fat to water ratios in raw milk could have contributed significantly to the dilution problem, especially in the samples with low concentrations of SMR. Also, the layered raw milk system may have prevented the SMR (associated with the predominantly fat phase) from competing with bound SMR (competitor conjugate) for antibody at the surface of the microtiter plate (predominantly aqueous phase). Although there were problems in preparing the standard curves using raw milk, the curves that were obtained were virtually the same as those prepared using homogenized milk except for the high uncertainty found for the lowest levels of SMR. Other advantages to the use of homogenized milk were that it provided a consistent fat content in the milk from trial to trial, and was a composite sample from many cows.

Sulfonamide EIA Cross-reactivity. Sulfonamide cross-reactivity studies were performed for each of the competitor conjugates using homogenized milk spiked with the sodium salts of SMR, SMT, STZ and SDX (Figure 3.1). Although SDX was not present in the 3-Sulvit, it is the only sulfonamide which is approved for use in lactating cows (Cullor and Chen, 1991). It was also important to evaluate SDX for SMR EIA cross-reactivity beacuse it is a sulfonamide that can be detected by both the CITE Sulfa Trio and the Charm II assays. The results of the EIA cross-reactivity tests are reported in Table 3.3.

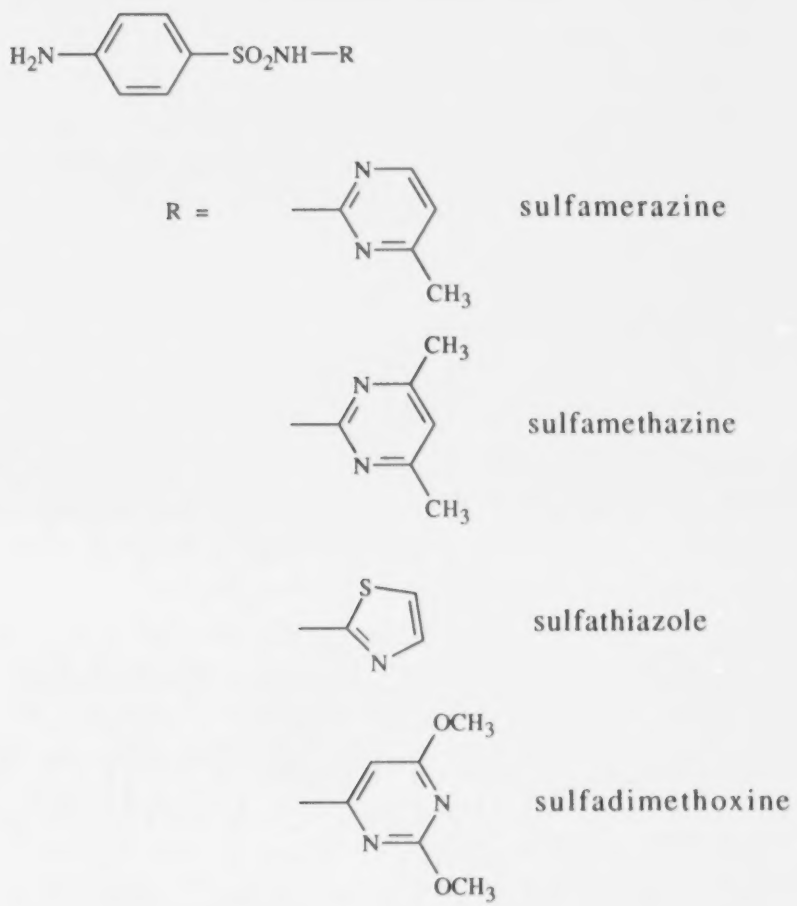

Figure 3.1. Structures of Sulfonamides.

Table 3.3. Cross-reactivity of Sulfonamides with EIA Competitor Conjugates

Sulfonamide	SMR-BSA(L) I_{50} (nM)	SMR-BSA(L) cross-reactivity[a] (relative %)	SMR-SA-BSA(L) I_{50} (nM)	SMR-SA-BSA(L) cross-reactivity[a] (relative %)
Sulfamerazine	5.2	100	11	100
Sulfamethazine	97	5.4	227	4.8
Sulfathiazole	1655	0.31	1803	0.61
Sulfadimethoxine	>10000	<0.02	4816	0.23

[a] calculated from the corresponding I_{50} value (nM) for each conjugate

Both competitor conjugates demonstrated that the polyclonal antibodies had a strong specificity for SMR. Little affinity for STZ, and especially SDX, was demonstrated by the antibodies. Even the structurally similar sulfonamide, SMT, showed only a 5.4% or less cross-reactivity when compared with the same molar ratio of SMR. Other authors have found 30% (Fleeker and Lovett, 1985), 12.1% (Singh *et al.*, 1989) or 10% (McCaughey *et al.*, 1990) cross-reactivity with antisera produced against SMT when tested with samples containing SMR. Antibodies prepared against SMT may find it easier to accommodate the smaller SMR into their binding sites, whereas SMR specific antibodies may find it more difficult to fit the extra methyl group of SMT into their binding sites.

3-Sulvit Treated Cows. 3-Sulvit is a mixture of sulfonamides, vitamins, and electrolytes for the treatment of bacterial infections of swine, sheep and cattle. Although the product was easily obtained from a local pharmacy without a prescription, a fine print warning on the package states that 3-Sulvit treatment should not be given to animals within 10 days of slaughter, and that it should not be administered to lactating dairy animals. The obvious reason for this warning is that high levels of sulfonamide residue will be found in the meat or milk soon after the intake of 3-Sulvit by the animal. Using 3-Sulvit as a way of introducing sulfonamides into the cows' milk was desirable for test evaluations, especially in light of our difficulty in spiking raw milk at very low sulfonamide levels. The administration of 3-Sulvit would also allow for evaluation of the effect of sulfonamide metabolites on test performance. Various sulfonamide metabolic products are formed by the cow, but commonly sulfonamides are acetylated at the N^4-position (Rehm *et al.*, 1986; Medina *et al.*, 1992; Paulson *et al.*, 1992b). Paulson *et al.* (1992a) also reported that a major metabolite in milk can result from lactose conjugation at the N^4-position of the sulfonamide. Because the immunogen used in this study was produced through conjugation to the carrier protein at the N^4-position, the EIA should be able to recognize the N^4 altered metabolites of SMR. The ability of antibody-based tests to recognize N^4 sulfonamide metabolites has been reported by Sheth *et al.* (1990) and Medina *et al.* (1992).

Analysis of SMR EIA Conditions

The production and characterization of the azo- and hemisuccinate-linked competitor conjugates was discussed in chapter 2. During that study, various hapten-protein molar ratios were examined with two different linking methods, and the conditions of the EIAs optimized to accommodate all six conjugates with aqueous SMR dilutions. This study, however, required milk dilutions of SMR for the preparation of standard curves. Using homogenized milk rather than water for preparing the SMR standard curves resulted in an overall decrease in the maximum absorbances (fewer antibodies binding to the competitor

conjugates), but improved SMR detection capabilities for all conjugates. This effect has been noted in other systems (Assil and Sporns, 1991), but since homogenized milk is a complex mixture of fats, proteins, sugars and salts, it was still surprising that the detection capabilities of the competitor conjugates were not decreased due to the presence of all of these potentially interfering compounds. Improved EIA detection capabilities in a food system, such as our milk EIAs, may be explained by the selection of antibodies with higher binding affinities for SMR in the polyclonal antiserum. The lower affinity antibodies in the antiserum may experience increased interference from the milk components.

For this study, only SMR-BSA(L) and SMR-SA-BSA(L) were chosen for further evaluation. These two competitor conjugates had the best detection levels, similar hapten-protein molar ratios, but different linking arms. The emphasis was switched from a comparison of the competitor conjugates under similar conditions to attain the maximum performance from each conjugate under its own optimal conditions with SMR homogenized milk dilutions. Under the newly optimized conditions, it was found that the hemisuccinate-linked competitor conjugate (SMR-SA-BSA(L)) required half the concentration for coating of the microtiter plate and polyclonal rabbit serum as the azo-linked competitor conjugate (SMR-BSA(L)) to produce approximately the same absorbance values. Also, the SMR-BSA(L) conjugate still maintained the lower detection limit. These two findings seemed to contradict what might be logically expected. Because the polyclonal antibodies were prepared using an azo-linked immunogen, it is presumed that although a different carrier protein was used, more antibodies would have compatible binding sites for SMR-BSA(L) than SMR-SA-BSA(L). Therefore, if the ability of the carrier protein (BSA) to bind to the microtiter plate was equal for both competitor conjugates, the SMR-BSA(L) competitor conjugate should have required a smaller concentration for coating the microtiter plate, and less antibody in solution, than the SMR-SA-BSA(L) competitor conjugate. This was not the case. Also, it was presumed that by changing the linking arm of the immunogen and the competitor conjugate, the bias for plate bound hapten would decrease, resulting in a more evenly balanced competition, and a lower detection limit for the SMR-SA-BSA(L) conjugate. This too was not the case as the SMR-BSA(L) conjugate was the most sensitive. The answer to these contradictions may be in the nature of the azo-linked competitor conjugate.

Since Landsteiner and Van der Scheer's (1936) experimentation with azo-linked haptens, problems of using an azo linking method for the immunogen and the competitor conjugate seem negligible. It may be the rigidity of the azo linkage which aids in reversible binding and improved competition. The flexible arm of the hemisuccinate linkage may lead to a more stable antibody-antigen interaction for the plate bound hapten, resulting in less

effective competition by free hapten. Also, more plate bound hapten may be exposed by this flexible arm, resulting in an increased concentration of antibody bound to the plate.

Analysis of Sulfonamide-contaminated Raw Milk Samples by SMR EIA, Charm II Assay, and CITE Sulfa Trio Test

The results of the analyses for the three test cows' milk samples are focused around the detection of sulfonamide in the milk at the 10 ppb level, because this was the level which the CITE Sulfa Trio and Charm II microbial receptor assays have designated as a control point. Within Tables 3.4, 3.5 and 3.6 are the results of the milk analyses for cows 8421, 8881 and 9020, respectively. Day 1/am milk for all cows, as indicated by the Charm II assay, contained less than 10 ppb of sulfonamide. Within approximately 5 h of treatment (day 1/pm), all cows had sulfonamide in their milk above the 10 ppb level, as determined by the Charm II assay and SMR EIAs. Examination of results from day 1/pm until day 7/am demonstrated the sulfonamide detection capabilities of each test.

For the SMR EIAs there was a measurable decrease in SMR content of the milk from day 3/pm to day 7/am. Taking into consideration only the ppb values determined from the linear regions of the standard curves (numerical results <100 ppb and not proceeded by "~") it was found that there was considerable agreement between the azo- and hemisuccinate-linked competitor conjugates as to the amount of SMR in the milk near and below the 10 ppb range, for cows 8421 and 8881. However, slightly less agreement as to the SMR concentration of the milk, determined by the two competitor conjugates, was observed for cow 9020.

The CITE Sulfa Trio test was used to determine SMT and STZ contents of the cows' milk. Although the CITE test will also determine SDX, this sulfonamide was not present in 3-Sulvit, and the test spots for SDX were consistently negative in tests with the cows' milk samples. The CITE Sulfa Trio test was used to determine the sulfonamide content of the milk through the examination of a range of milk samples that were known to have a 10 ppb sulfonamide concentration by Charm II analysis. At times it was possible to distinguish between the concentration of STZ and SMT through the intensity of the spot that was formed. This provided the results at day 4/pm for 8421 and 8881, where the SMT spot could be reported as being >10 ppb while the STZ spot was <10 ppb.

The operator's manual for the Charm II assay sets the criteria for a safe level control point for determining negative samples, as the control point (which was determined to be 1100 cpm) plus 15% (= 1265 cpm). For the purposes of this study, there was only an interest in quantifying the sulfonamide concentration in the milk at a 10 ppb level,

Table 3.4. Results of 3-Sulvit Treatment of Cow 8421

Milking (8421)		SMR-BSA(L) EIA	SMR-SA-BSA(L) EIA	CITE Sulfa Trio	Charm II assay	
day	hours	(ppb)	(ppb)	(ppb)[a]	(cpm)[b]	(ppb)[b]
1/am	0	[c]	[c]	—	1431	<10
1/pm	9	>100	>100	>10	789	>10
2/am	24	>100	>100	—	828	>10
2/pm	33	>100	>100	—	959	>10
3/am	48	>100	>100	—	856	>10
3/pm	57	~29[d]	~73	>10	926	>10
4/am	72	5.2	5.4	—	857	>10
4/pm	81	3.5	3.7	>10 (SMT only)	996	>10
5/am	96	1.1	1.1	≈10	1056	>10
5/pm	105	0.64	0.73	<10	1062	>10
6/am	120	[e]	[e]	[e]	[e]	
6/pm	129	0.41	~0.24	—	1826	<10
7/am	144	0.16	~0.10	—	1700	<10

[a] the CITE Sulfa Trio test is able to detect <, >, or ≈10 ppb of SMT, STZ, SDX
[b] 1100 cpm ≈10 ppb concentration of sulfonamide in the milk
[c] sulfonamide-free milk samples for SMR EIA
[d] "~" quantifiable results that fall outside the linear region of the sigmoidal standard curves
[e] sample was inadvertently discarded prior to analyses

Table 3.5. Results of 3-Sulvit Treatment of Cow 8881

Milking (8881) day	hours	SMR-BSA(L) EIA (ppb)	SMR-SA-BSA(L) EIA (ppb)	CITE Sulfa Trio (ppb)[a]	Charm II assay (cpm)[b]	(ppb)[b]
1/am	0	[c]	[c]	—	2504	<10
1/pm	9	>100	>100	—	946	>10
2/am	24	>100	>100	—	976	>10
2/pm	33	>100	>100	—	864	>10
3/am	48	>100	>100	—	931	>10
3/pm	57	~38[d]	>100	—	934	>10
4/am	72	~20	~40	>10	987	>10
4/pm	81	~11	~18	>10 (SMT only)	1024	>10
5/am	96	5.9	6.5	≈10	1052	>10
5/pm	105	6.9	6.8	<10	1175	<10
6/am	120	1.7	2.4	—	1156	<10
6/pm	129	1.4	2.2	—	1270	<10
7/am	144	0.31	~0.46	—	1373	<10

[a] the CITE Sulfa Trio test is able to detect <, >, or ≈10 ppb of SMT, STZ, SDX
[b] 1100 cpm ≈10 ppb concentration of sulfonamide in the milk
[c] sulfonamide-free milk samples for SMR EIA
[d] "~" quantifiable results that fall outside the linear region of the sigmoidal standard curves

Table 3.6. Results of 3-Sulvit Treatment of Cow 9020

Milking (9020) day	hours	SMR-BSA(L) EIA (ppb)	SMR-SA-BSA(L) EIA (ppb)	CITE Sulfa Trio (ppb)[a]	Charm II assay (cpm)[b]	(ppb)[b]
1/am	0	[c]	[c]	—	2234	<10
1/pm	9	>100	>100	—	814	>10
2/am	24	>100	>100	—	923	>10
2/pm	33	>100	>100	—	940	>10
3/am	48	~25[d]	>100	>10	837	>10
3/pm	57	~11	~44	>10	936	>10
4/am	72	3.3	8.2	>10	1023	>10
4/pm	81	2.8	7.7	≈10	1218	<10
5/am	96	0.89	2.1	<10	1239	<10
5/pm	105	1.2	2.1	—	1334	<10
6/am	120	0.24	1.4	—	1918	<10
6/pm	129	0.19	~0.59	—	1993	<10
7/am	144	~0.14	~0.58	—	2529	<10

[a] the CITE Sulfa Trio test is able to detect <, >, or ≈10 ppb of SMT, STZ, SDX
[b] 1100 cpm ≈10 ppb concentration of sulfonamide in the milk
[c] sulfonamide-free milk samples for SMR EIA
[d] "~" quantifiable results that fall outside the linear region of the sigmoidal standard curves

therefore, 1100 cpm was chosen as the control point. This meant that values of <1100 cpm represented a concentration of sulfonamide in the milk of >10 ppb, and values of >1100 cpm represented a sulfonamide concentration of <10 ppb. By day 7/am, results of the Charm II indicated that all cows' milk was below the 10 ppb level even with the 15% safety factor.

Considering that there were three individual cows and four test situations used to examine the sulfonamide-contaminated milk samples, all test results were in relative agreement as to the date when the sulfonamide concentration in the milk samples fell below 10 ppb, or a 10 ppb cut-off date (Table 3.7). For cow 8421, the findings by Charm II assay of day 6/pm, being the first milk <10 ppb, are probably exaggerated. However, since day 6/am milk was not available, determinations had to be made based on day 6/pm results. The different daily concentrations of sulfonamides in the milk samples may be attributed to three factors. First, there were the different levels of the three sulfonamides found in 3-Sulvit. Because SMR was the sulfonamide in the smallest proportion in 3-Sulvit, it was logical that the SMR EIAs would show the quickest reduction in sulfonamides, followed by the other two tests. Second, metabolic factors can influence the rate of excretion of sulfonamides into milk. Rehm *et al.* (1986) reported that there are considerable variations in the rates of sulfonamide excretion between individual animals of the same species. Also, a the state of ionization of the sulfonamide has been found to influence its excretion rate (Rasmussen, 1958). The third and final factor was the variation of sulfonamide sensitivity of each analyses. For example, the operator's manual for the Charm II assay reports that the assay is 2.5 times more sensitive to SMR and STZ, than to SMT, for which the 10 ppb level was established. This increased sensitivity to SMR and STZ for the Charm II assay would have prolonged the 10 ppb cut-off date.

The structural similarities between SMR and SMT, and the availability of SMR in commercially produced sulfonamide preparations, may make future determinations of residual SMR in milk a pressing concern. This study has developed two SMR EIAs which have the potential to be sensitive analytical methods for the detection of SMR in milk.

Table 3.7. Identification of a 10 ppb Cut-off Date for Milk Samples using Sulfonamide Assays

	SMR-BSA(L) EIA	SMR-SA-BSA(L) EIA	CITE Sulfa Trio		Charm II assay
	(sulfamerazine)[a]	(sulfamerazine)	(sulfamethazine)	(sulfathiazole)	(all sulfonamides)
Cow 8421	4/am	4/am	5/am	4/pm	6/pm
Cow 8881	5/am	5/am	5/am	4/pm	5/pm
Cow 9020	4/am	4/am	4/pm	4/pm	4/pm

[a] sulfonamide(s) detected by assay

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Chapter: 4

AN INVESTIGATION INTO THE PREPARATION OF A MONOCLONAL ANTIBODY FOR MULTIRESIDUE SULFONAMIDE DETECTION

INTRODUCTION

Sulfonamides are a group of antimicrobial compounds whose bacteriostatic properties are used to control infections in humans and animals (Steele and Beran, 1984). The use of sulfonamides in animal husbandry has led to the problem of residual contamination, or concern for the possibility of contamination, in food products derived from the flesh of hogs (Fischer *et al.*, 1992; Singh *et al.*, 1989; Dixon-Holland and Katz, 1988), veal (Wilson *et al.*, 1991; Barnes *et al.*, 1990), poultry and eggs (Horii *et al.*, 1990; Agarwal, 1992), fish (Pleasance *et al.*, 1991; Uno *et al.*, 1993), milk from dairy cattle (Larocque, *et al.*, 1990; Charm *et al.*, 1988) and honey from bees (Sheth and Sporns, 1990). The concern over sulfonamide contamination of foods stems from the health risks associated with both short term (allergy and acute toxicity) and long term (cancer) sulfonamide ingestion (Rehm *et al.*, 1986). Recently, milk has been the focus of many screening tests developed in an attempt to control undesirable sulfonamide intake. With the exception of the Charm II assay and Charm Cowside, screening tests that have been developed with multiple sulfonamide capabilities lack the ability to detect more than a few sulfonamides at or below a 10 part per billion (ppb) level in milk (Bishop *et al.*, 1991). A 10 ppb level has been reportedly established by the United States Food and Drug Administration as a safe level for all sulfonamides (Zomer *et al.*, 1992).

Immunoassays are valuable analytical tools for the detection of a variety of compounds in foods (Gazzaz *et al.*, 1992). Commercial immunoassays have been produced for individual sulfonamides, particularly sulfamethazine (Medina *et al.*, 1992), but an immunoassay with a multiple sulfonamide detection capability is yet to be marketed. Previous research performed by Sheth and Sporns (1991) and Assil *et al.* (1992) has shown that polyclonal antiserum may be produced with the ability to detect a number of sulfonamides. Sheth and Sporns (1991) demonstrated that a population of antibodies in antiserum, with multiple sulfonamide detection capabilities, could be selected for use in a competitive indirect immunoassay by careful choice of the competitor conjugate used to coat the wells of the microtiter plate. Also, Assil *et al.* (1992) found that affinity purification, in conjunction with the careful choice of the competitor conjugate, was even more effective in isolating the desired antibodies from the polyclonal antiserum. Affinity purification removed the background interference caused by high affinity antibodies, and improved the detection capabilities of the immunoassay. With the maximum potential of the polyclonal antiserum achieved through affinity purification and competitor conjugate manipulation, this study describes the attempt at the production of monoclonal antibodies (MAbs) with multiple sulfonamide binding capabilities.

EXPERIMENTAL

Reagents

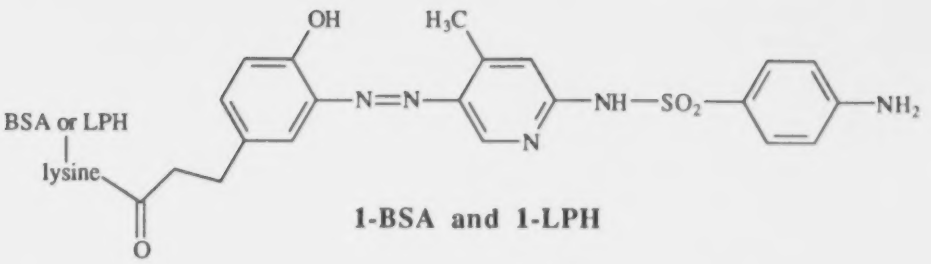
Sodium sulfaquinoxaline (SQX), sodium sulfadimethoxine (SDX), sodium sulfamethazine (SMT), sodium sulfathiazole (STZ), sulfanilamide (SNM), phenyl-ring-UL-^{14}C sulfamethazine (^{14}CSMT), thimerosal, Tween 20, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB), oxalacetic acid, D,L dithiothreitol, bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St. Louis, MO). Bromophenol blue (water soluble) was obtained from BDH Chemicals Ltd. (Poole, England). Ammonium sulfate (enzyme grade) was supplied by Fisher Scientific (Ottawa, ON). Urea peroxide, goat anti-mouse IgM peroxidase-conjugated antibodies and goat anti-mouse IgG peroxidase-conjugated antibodies were obtained from Calbiochem Corp. (La Jolla, CA). Acrylamide (electrophoresis grade) and 50% PEG (1500) in 75 μM HEPES buffer were ordered from Boehringer-Mannheim Canada Ltd. (Laval, PQ). Low melting point agarose (electrophoresis grade) and sodium pyruvate were from Gibco BRL (Burlington, ON). All other chemicals used were of reagent grade or better.

The following were purchased as sterile solutions from Gibco BRL (Burlington, ON): L-glutamine supplemented Roswell Park Memorial Institute (RPMI) 1640 medium, Hybridoma SFM (hybridoma serum-free medium), L-glutamine (200 mM), fetal bovine serum and calf serum. The fetal bovine serum and calf serum were heat inactivated (56°C for 30 min) before use. Supplied together in a solution were penicillin and streptomycin (10,000 units/mL sodium penicillin G and 10,000 μg/mL streptomycin sulfate). Also supplied together as a single solution were hypoxanthine and thymidine (100 x liquid). Aminopterin was purchased in the form of a 100 x sterile powder which was reconstituted with water.

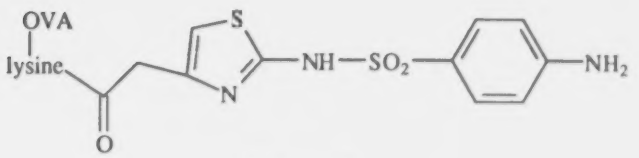
Phosphate-buffered saline (PBS) was prepared from sodium chloride (154 mM), disodium hydrogen phosphate (7.8 mM), potassium dihydrogen phosphate (2.2 mM), and thimerosal (0.247 M), pH adjusted to 7.3 with sodium hydroxide and hydrochloric acid. PBST was prepared from PBS with the addition of Tween 20 (0.05%, w/v) before pH adjustment.

Immunization

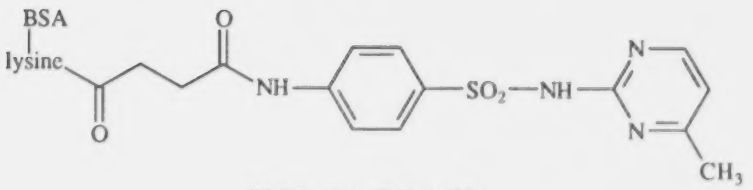
The synthesis of the conjugate used in the immunizations, an amide-linked, N^1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide-*Limulus polyphemus* hemolymph conjugate (1-LPH), was described by Assil *et al.* (1992). The structure of 1-LPH is given in Figure 4.1.



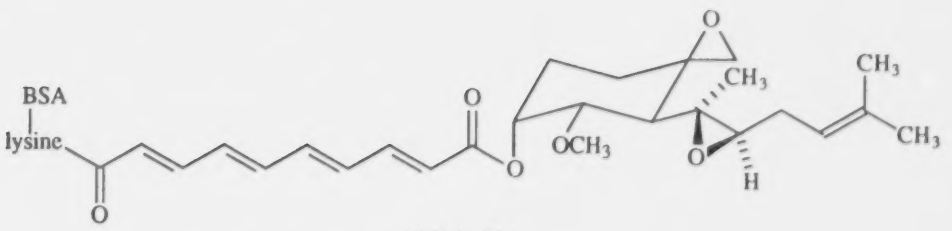
1-BSA and 1-LPH



OVA-TS



SMR-SA-BSA(H)



BSA-FA

Figure 4.1. Structures of Competitor and Injection Conjugates.

The immunogen was prepared using the Ribi Adjuvant system for mice (Cedarlane Laboratories Ltd., Hornby, ON) to which was added 2.2 mL of a 0.3 mg 1-LPH per mL sterile saline solution. Injections of the mice were made at 2 sites, 0.1 mL subcutaneous and 0.1 mL intraperitoneal. The first boost of the mice was made 21 days after the initial injection, and boosts were administered on days 35, 42, 49, 63 and 98. Bleeds from the tail of the mice (200 µL) were taken on days 26 and 69, and the blood was processed using Microtainer brand serum separators tubes (Becton Dickinson and Co., Rutherford, NJ). By day 101 mice had been sufficiently immunized for a fusion.

Approximately 9 months later, a second set of injections was performed on the mice remaining after the first round of immunizations. 1-LPH (0.38 mg/mL sterile saline) was prepared for injection using the Ribi Adjuvant system for mice, and was administered using the previously described injections sites and inoculation amounts. Beginning on day 342 from the last immunization, injections were made once a week for three weeks. Bleeds from the tail of the mice were taken on day 356 for testing. These test bleeds indicated that additional immunizations did not warrant a second fusion.

Myeloma Cell Line and Media

The myeloma cell line NS-1, a non-secreting clone of P3x63Ag8 (American Type Culture Collection, Rockville, MD), was used for the production of hybridomas. Several different media formulations were required for myeloma cell culture, and hybridoma cell culture and selection. Serum-free RPMI was prepared from L-glutamine supplemented RPMI 1640 medium, which was further fortified with additional L-glutamine to a concentration of 2 mM. Also added were penicillin (100 units/mL), streptomycin (100 µg/mL), oxalacetic acid (0.5 mM) and sodium pyruvate (1 mM). Complete RPMI was prepared by adding fetal bovine serum to serum-free RPMI at a concentration of 20% (v/v). A serum reduced form of complete RPMI (complete RPMI(SR)) was prepared substituting calf serum for fetal bovine serum at a 10% (v/v) concentration. HT medium was complete RPMI plus sodium hypoxanthine (100 µM) and thymidine (16 µM). HAT medium was prepared from HT medium by adding aminopterin to a final concentration of 0.4 µM. Complete HSFM was prepared by fortifying Hybridoma SFM with L-glutamine, sodium pyruvate, and oxalacetic acid to the same final concentration as the serum-free RPMI. Pencillin and streptomycin were added to complete HSFM at 1/10 of the serum-free RPMI concentration. Conditioned medium was prepared by filtering complete RPMI (which had formerly sustained the growth of NS-1 cells) through a 0.2 µm filter. The filter sterilized medium was further fortified with L-glutamine (2 mM), penicillin (50 units/mL), and streptomycin (50 µg/mL).

Cell Fusion and Selection

Harlow and Lane (1988) and McCullough and Spier (1990) were used as general references for hybridoma production and manipulation with some procedural modifications.

Prior to fusion, the NS-1 cells were cultured in complete RPMI using sterile 25 and 75 cm^2 tissue culture flasks (Corning Inc., Corning, NY) and a 37°C, CO_2 incubator (7% CO_2 and 90-100% humidity). Cell density was maintained between 0.5-3 x 10^5 cells per mL by subculturing every 2-3 days. On the day of fusion, the actively dividing myeloma cells were combined from several growth flasks into a 50 mL conical sterile centrifuge tube (Corning Inc., Corning, NY). Cells from each growth flask were pelleted (250 x *g* for 5 min), the complete RPMI decanted and save for later use, and the cells transferred in 5 mL of serum-free medium to a single centrifuge tube. The complete RPMI decanted from the myeloma cells was filtered through a 500 mL, 0.2 µm cellulose acetate bottle top filter (Costar Corp., Cambridge, MA) into a 500 mL sterilized glass bottle. This medium was then prepared for use as conditioned medium in the cloning procedure. The myeloma cells in the centrifuge tube were washed (pelleted and resuspended) twice using 10 mL of serum-free medium per wash. After the second washing, the myeloma cells in serum-free RPMI were counted using a hemocytometer, and stored in a 37 °C, CO_2 incubator until the time of the fusion.

On the day of fusion, the desired mouse from titer determinations was asphyxiated using CO_2, and the spleen was aseptically removed and placed in a sterile petri dish containing 10-15 mL of serum-free RPMI. Cells were removed by making small tears in the spleen sac, followed by repeated injections of the surrounding serum-free RPMI into the sac, using two sterile 21 gauge needles attached to two sterile 5 cc syringes. Once the sac was sufficiently void of cells, the splenocytes were transferred to a 50 mL centrifuge tube and washed three times with 10 mL serum-free RPMI. During the first washing, red blood cells were lysed with the addition of 4 mL of 0.8% ammonium chloride (incubated at room temperature for 3 min), before the addition of serum-free RPMI. After the third washing, a hemocytometer was used to determine the number of splenocytes in the 10 mL of serum-free RPMI.

Cell fusion was initiated by mixing the splenocytes and myeloma cells in a ratio of 6.7:1, respectively. The serum-free RPMI was removed after centrifugation at 800 x *g* for 5 min. A 1 mL aliquot of 50% PEG 1500 in HEPES buffer was added to the mixture of splenocytes and myeloma cells (with stirring) over a period of 1 min. The mixture was then stirred for an additional 1 min. During the next 2 min, 2 mL of serum-free RPMI was added (with stirring) at a rate of 1 mL per min. This was immediately followed by the

addition of 8 mL of serum-free RPMI (with stirring) over the next 3 min. The supernatant was decanted after centrifugation (250 x *g* for 5 min), and the cells resuspended in 50 mL of complete RPMI. The cells and medium were transferred to a tissue culture flask and left overnight in the 37 °C, CO_2 incubator. The next day the complete RPMI was removed by centrifugation (250 x *g* for 5 min), decanting and discarding the medium, and the cells resuspended in 50 mL of HAT medium. The cells in HAT medium were distributed in 150 µL aliquots to the wells of 6 sterile 96 well plates (ICN Biomedicals Canada Ltd., Mississauga, ON). Three days after the fusion, and before the first subculture with HAT medium, the number of wells positive for hybridoma growth were recorded. The cells were subcultured in HAT medium every 2-3 days until the 11th day after fusion. At this time wells that screened positive by enzyme immunoassay (EIA) for desired antibody were subcultured into HT medium in the wells of a 24 well plate (ICN Biomedicals Canada Ltd., Mississauga, ON).

Cell Cloning

Cells were cloned from the 24 well plate by limiting dilution into soft agar. Prior to the day of cloning, a 2.4% (w/v) solution of low melting point agarose in 0.15 M NaCl was prepared, sterilized by autoclaving, then stored at 4°C. On the day of cloning, the agarose was melted and diluted to 0.48% (w/v) with conditioned medium. A 0.5 mL aliquot was taken from the well of the 24 well plate chosen for cloning, and six, 1:1 serial dilutions of the cells were made with conditioned medium. A 100 µL sample of each serial dilution was added to 2 mL of the 0.48% agarose, and after vigorous mixing, the cells in agarose were distributed into 4 wells (in a single column) of a 24 well plate.

After 7-10 days of growth in the soft agarose, individual cell clumps derived from the division of a single hybridoma were transferred from the agarose to 100 µL of conditioned medium in the wells of sterile BBL Minitek plates (Becton Dickinson and Co., Cockeysville, MD). The transfer was performed using an inverted phase microscope in a laminar flow hood, and a mouth pipetting apparatus. The mouth pipetting apparatus consisted of sterile 25 µL disposable borosilicate micropipet and micropipet holder, connected to a micropipet mouth piece via approximately two feet of sterilized latex tubing (3.2 mm inner diameter). Within the two foot length of tubing were inserted two, 0.22 µm Millex-GS filters (Millipore Corp., Bedford, MA). Hybridomas multiplying in the conditioned medium of the wells of the BBL Minitek plates were subcultured into complete RPMI. If screening EIAs determined their supernatant samples were positive for the desired antibody, the hybridomas were transferred from the minitek plate to a 24 well plate. From the 24 well plate the cloning process was performed a second time to ensure the

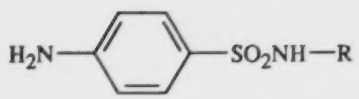
stability of the cell line. The isotype of the MAb produced by each hybridoma clone was determined using a Mouse Monoclonal Ab Isotyping Kit (ISO-1) from Sigma Chemical Co. (St. Louis, MO)

Competitive Indirect and Screening EIA Procedures

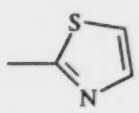
Each well of a 96-well Immulon 2 flat-bottomed microtiter plate (Dynatech Laboratories Inc., Chantilly, VA) was coated with either 200 µL/well of 1% BSA in PBS solution or 200 µL/well of a 6 to 7 ppb solution (w/v) of one of two competitor conjugates. The choice of competitor conjugates were: an amide-linked, N^1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide-bovine serum albumin conjugate (1-BSA) or an amide-linked, N^1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide-ovalbumin conjugate (OVA-TS), dissolved in PBS. The synthesis of 1-BSA is described by Assil *et al.* (1992) and the synthesis of OVA-TS by Sheth and Sporns (1991). The structures of both of these competitor conjugates are given in Figure 4.1. The plate was protected from evaporation with an acetate plate sealer and left overnight at 4°C. The next day the coating solution was shaken from the wells and the entire plate coated with 200 µL per well of 1% BSA in PBS. The plate was sealed with a plate sealer and left to incubate for 1 h at room temperature. After 1 h, the 1% BSA in PBS solution was shaken from the plate and the wells washed three times with PBST, shaking and blotting the PBST from the plates onto paper towels between each washing. At this stage the plates were ready for use in a screening or competitive EIA.

For a screening EIA, 100 µL per well of hybridoma supernatant or water (control) was added to the plates, which were then sealed and incubated for 2 h. For a competitive EIA, aqueous dilutions of one or more of the sulfonamides, SNM, STZ, SMT, SDX, or SQX (Figure 4.2) were added (100 µL/well) as a source of free hapten in solution. To some of the wells, 100 µL of water was added as a control. Hybridoma supernatant or reconstituted freeze-dried MAb (20 µg lyophilized material/mL 0.05% BSA in PBST) was added to all wells of the competitive EIA plate (100 µL/well) and the plate sealed and incubated for 2 h.

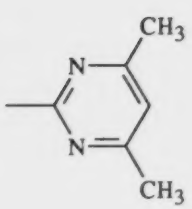
Following the 2 h incubation, both the screening and competitive EIA plates were developed in the following manner. The solution was shaken from the wells and the plate was washed and blotted 3 times as previously described. In early screening tests and competitive EIAs both goat anti-mouse IgM peroxidase-conjugated antibodies (diluted 10,000 times with PBST) and goat anti-mouse IgG peroxidase-conjugated antibodies



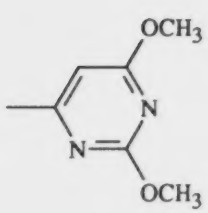
R = —H sulfanilamide



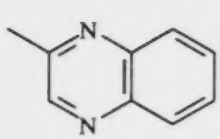
sulfathiazole



sulfamethazine



sulfadimethoxine



sulfaquinoxaline

Figure 4.2. Structures of Sulfonamides.

(diluted 6000 times with PBST) were added to individual wells (200 µL/well) of the microtiter plate. Once the hybridomas were serotyped as IgM, only the goat anti-mouse IgM antibodies were used. After the addition of the peroxidase-conjugated antibodies, the plate with plate sealer was incubated for 2 h. Following the incubation, the plate was washed with PBST as before, and 200 µL of a substrate solution (TMB (0.1 mg/mL) and urea peroxide (1.0 mg/mL) dissolved in 0.1 M citrate buffer, pH 4.0) was added to each well. After sufficient color had developed from the enzyme-substrate reaction (approximately 0.2-0.8 absorbance units), the reaction was stopped with the addition of 50 µL/well of 2.0 M sulfuric acid, and the absorbance read at 450 nm.

MAb Collection and Purification

Purification of MAb was accomplished through the growth of cloned hybridoma lines on complete HSFM, followed by ammonium sulfate precipitation of the MAb from the supernatant, then dialysis. Hybridomas were adapted to growth on complete HSFM by a two step procedure. Actively growing cell lines were subcultured from complete RPMI containing 20% fetal bovine serum, to complete RPMI(SR). Hybridomas adapted to the complete RPMI(SR) were grown to a density of 1-2 x 10^5 cells per mL. The hybridoma cell lines were then subcultured from complete RPMI(SR), to complete HSFM, through repeated 1:1 dilutions with complete HSFM until the medium was essentially complete HSFM. Once it was determined that the hybridomas were successfully adapted to growth in complete HSFM, they were prepared for growth as a "suicide culture" based on the procedure described by Seaver (1992).

Cells actively growing in complete HSFM were resuspended in fresh complete HSFM at a cell density of 1-2 x 10^5 cells per mL. The cells were then grown until the cell viability was less than 10% (suicide culture), as determined using trypan blue exclusion as a test of cell viability (Harlow and Lane, 1988). At the end of their growth period, suicide cultures attained a cell density which was consistently between 7-8 x10^5 cells/mL. Cells were removed from the medium by centrifugation, and the supernatant collected in a 250 mL centrifuge bottle. The MAb was then extracted from the medium by precipitiaton with ammonium sulfate.

Using the table supplied in Harlow and Lane (1988), ammonium sulfate was added to the supernatant from the suicide culture to a concentration of 60%. Once the ammonium sulfate was dissolved, the supernatant was slowly stirred for 30 min at room temperature. At the end of the 30 min, the precipitated protein was pelleted (maximum force of 24,000 x *g* for 30 min). The supernatant was decanted into a second 250 mL centrifuge bottle and the pellet resuspended in 5 mL of 0.1 M ammonium bicarbonate (pH adjusted to 7.9 with

ammonia). A second protein fraction was collected by raising the ammonium sulfate concentration (in the 60% supernatant) to 80%. The 60-80% supernatant was stirred, centrifuged, and protein pellet was resuspended in the same manner as the 0-60% fraction. The resuspended protein pellets from the 0-60% and 60-80% fractions were transferred individually to Spectra/Por 7 (50,000 molecular weight cut-off) membrane tubing (Spectrum Medical Industries Inc., Los Angeles, CA). Each fraction was diaylzed twice against 1 L of 0.1 M ammonium bicarbonate (pH 7.9) for 24 h, and then the contents of the membrane tubing were freeze-dried. The lyophilized material was stored at 4°C.

MAb Quantification

The Bio-Rad protein assay (Bio-Rad Laboratories Canada Ltd., Mississauga ON) and ultraviolet (UV) spectrophotometric methodology were used to estimate the MAb production from the hybridomas grown in complete HSFM. The Bio-Rad protein assay was used to determine the MAb content in samples of suicide culture supernatants taken before ammonium sulfate precipitation, and of reconstituted lyophilized material collected after ammonium sulfate precipitation. A standard curve created from bovine IgG (supplied by Bio-Rad) was used to quantify the MAb concentration using the microassay procedure outlined by Bio-Rad for cuvets.

UV spectrophotometric determination of MAb content was performed only on dialyzed or reconstituted lyophilized material, from the ammonium sulfate precipitations. An HP 8452 A diode array spectrophotometer (Hewlett-Packard Canada Ltd., Mississauga, ON) was used to make the measurements required for MAb quantitation using equation (1).

$$\text{IgM (mg / mL)} = \frac{(A_{280\ nm} - A_{350\ nm})}{1.2} \qquad (1)$$

Equation (1) is a modified form of the equation for IgM spectrophotometric quantitation given in Harlow and Lane (1988). The absorbance of the samples at 280 nm was corrected for background interferences by subtracting sample absorbance at 350 nm.

SDS-PAGE Procedure for MAb Evaluation

Bio-Rad's Mini Protean II separation system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON) was used to prepare 1.5 mm gels used for protein separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE procedure was based on the gel electrophoresis described by Laemmli (1970) with some modifications. The separatory gel was prepared using 0.375 M Tris-HCl (pH 8.8) containing 10% acrylamide, 0.1% sodium dodecylsulfate (SDS), 0.05% ammonium persulfate, and 0.1% tetramethylethylenediamine (TEMED). The stacking gel was prepared using 0.125 M Tris-HCl (pH 6.8) and contained 4% acrylamide, 0.1% SDS, 0.05% ammonium persulfate, and 0.2% TEMED. To dissociate the MAb before loading onto the gel, a 1:1 mixture of MAb in solution and sample buffer was heated in boiling water for 2 min. The sample buffer consisted of 100 mM dithiothreitol, 2% SDS, 32% (w/v) glycerol, 0.1% bromophenol blue in 0.0625 M Tris-HCl buffer (pH 6.8). Gels were run using a Bio-Rad power supply (20 mA constant current) and the proteins stained using 0.1% Coomassie Brilliant Blue R-250 in 25% isopropanol and 10% acetic acid. Gels were destained using 30% ethanol and 7% acetic acid and then stored in 7% acetic acid.

Two standard preparations were used to determine the approximate molecular weights of proteins in gels prepared by SDS-PAGE. One standard preparation was a serum/plasma rabbit IgG (Calbiochem Corp., La Jolla, CA), obtained as a lyophilized powder and reconstituted in water. The other standard preparation was a high molecular weight standard mixture (for SDS-PAGE) purchased from Sigma Chemical Co. (St. Louis, MO). The standard mixture contained the following proteins with the approximate masses: myosin from rabbit muscle (205 kDa), ß-galactosidase from *Escherichia coli* (116 kDa), phosphorylase B from rabbit muscle (97.4 kDa), bovine plasma albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase from bovine erythrocytes (29 kDa).

MAb-^{14}CSMT Binding Experiment

A stock solution containing ^{14}CSMT dissolved in 0.1 N sodium hydroxide was prepared, and determined to contain radioactivity level (decays per minute, dpm) of 6,334,952 dpm/mL. To a microcentrifuge tube containing 0.69 mg of purified, lyophilized IgM MAb (from hybridoma cell line HSFM A1) dissolved in 1.2 mL of 0.1 M ammonium bicarbonate (pH 7.9), was added 100 µL of the ^{14}CSMT stock solution. To a second microcentrifuge tube, another 100 µL of the ^{14}CSMT stock solution was added to 3 mg of BSA dissolved in 1.3 mL of 0.1 M ammonium bicarbonate (pH 7.9). Both solutions in the microcentrifuge tubes were incubated for 16 h at room temperature and then transferred to individual dialysis chambers prepared from Spectra/Por 1 membrane tubing (6000-8000

molecular weight cut-off, purchased from Spectrum Medical Industries Inc., Los Angeles, CA) and dialyzed against 500 mL of 0.1 M ammonium bicarbonate (pH 7.9) for 24 h. At the end of the dialyses, the radioactivity (measured in dpm) of samples taken from outside and inside the membrane tubings were determined in triplicate. All radioactivity measurements were made in a LS 1801 Liquid Scintillation Counter (Beckman Instruments Inc., Mississauga, ON) using 20 mL plastic scintillation vial (Fisher Scientific, Ottawa, ON) and EcoLite+ scintillation cocktail (ICN Biomedicals Inc., Irvine, CA).

RESULTS AND DISCUSSION

This work describes the continuation of research in our laboratory directed at producing an immunoassay with multiresidue sulfonamide detection capabilities. The production of a multiple sulfonamide immunoassay has been aided by a structural region common to all sulfonamides; however, difficulties have arisen when trying to direct an immune response against the common region due to its small size. Sheth and Sporns (1991) described the production of an EIA capable of detecting nine commercially available sulfonamides. Seven of the nine sulfonamides required five part per million (ppm) or less of sulfonamide per assay (maximum concentration required was 22 ppm) to reduce the absorbance measured in the assay by 50%. Using a different immunogen, Assil *et al.* (1992) were able to increase the spectrum of sulfonamides that could be detected, and demonstrated through affinity purification that increased EIA sensitivity and decreased background absorbance levels were possible by isolating a desired population of antibodies from the polyclonal antiserum. With the maximum potential of the polyclonal antiserum exploited in the first two studies, it was determined feasible to attempt to produce a MAb using the immunogen produced by Assil *et al.* (1992).

Hybridoma Production and Selection

The fusion produced 192 wells that were positive for hybridoma growth out of 360 wells seeded (53%) determined 12 days post fusion. At this time, all 360 wells were screened for the presence of antibodies having affinity for 1-BSA, the BSA conjugated equivalent of the immunogen 1-LPH. From the 192 wells that were positive for hybridomas, 27 screened positive for 1-BSA binding and were transferred to two 24 well plates. At the 24 well plate stage, hybridomas were evaluated using 1-BSA and OVA-TS as competitor conjugates for coating the wells of the microtiter plate, and serial dilutions of an aqueous mixture of five sulfonamides. The sulfonamide mixture contained SNM, STZ, SMT, SDX and SQX, and aqueous dilutions of the mixture had total sulfonamide concentrations ranging from 5000-0.5 ppb. OVA-TS was employed as a second competitor conjugate to restrict any preferential antibody binding to the linking arm that may arise with the use of 1-BSA. OVA-TS provided a different hapten from the immunogen, and was used successfully in competitive EIAs by Assil *et al.* (1992) when 1-BSA did not provide any competition. Competitive EIA tests were used to select 10 wells containing hybridomas from the 24 well plates for cloning. Wells that were chosen demonstrated at least a 20% reduction in absorbance with the addition of 0.5 ppm of mixed sulfonamide versus water. After the second cloning, three of the 10 hybridoma cell lines

taken from the wells of the 24 well plate remained. These cloned hybridomas were still producing MAb that demonstrated the same 0.5 ppm level of mixed sulfonamide competition when using 1-BSA or OVA-TS as competitor conjugates. At this point the competitive EIA tests were expanded to evaluate the sensitivities of the MAb from the three cloned hybridomas to each of the 5 sulfonamides in individual tests using only OVA-TS as the competitor conjugate. In earlier EIAs, OVA-TS had been the competitor conjugate which displayed the greatest degree of sulfonamide competition. A 20-50% reduction in absorbance was observed with all hybridomas when a solution of each sulfonamide in the range of 1-0.1 ppm was used in the competitive EIA.

Having prepared stable hybridomas producing MAbs which displayed promising levels of sulfonamide competition, efforts were directed towards determining of the affinities of the MAbs and increased MAb production. Serotyping tests showed that all three hybridomas were producing immunoglobulin M (IgM) MAb. For our purposes it was thought that a lower affinity IgM antibody (as opposed to a higher affinity IgG antibody) would be suitable since low affinity would be desirable for broad spectrum sulfonamide binding. It was felt that the promising level of competition observed using the IgM MAb could be improved if steps were taken to optimize the EIA. To provide enough purified IgM MAb for EIA optimization and sulfonamide competition experiments, hybridomas were grown on complete HSFM.

IgM MAb Collection and Purification

Complete HSFM was chosen for MAb production as it contained only insulin and transferrin as the defined proteins in the medium at a level of 20 μg/mL, thus simplifying downstream MAb purification. When adapting hybridomas to complete HSFM, it was found that one cell line would grow but would not produce antibody, and therefore that cell line was discontinued. The other two cell lines that grew and produced antibody were referred to as HSFM A1 and HSFM A6, and were prepared as suicide cultures for larger scale antibody collection. At this point cell lines were screened for MAb binding to OVA-TS and not for sulfonamide competition, as it was believed that earlier competitive EIAs had provided enough proof of the MAbs sulfonamide detection capabilities.

It was during the production of MAb from HSFM A1 and A6 suicide cultures that the problems associated with cold precipitation of IgM MAb were discovered. Supernatants from suicide cultures were initially stored at 4°C before the MAb was concentrated and collected by ammonium sulfate precipitation. During the cold storage it was observed that within 24 h, MAb would precipitate and collect at the bottom of the storage vessel. However, the MAb would appeared to redissolve when the supernatant

was warmed to 37°C. Morris and Inman (1968) reported of a similar occurrence when experimenting with IgM isolated from serum. The cold precipitation of protein in the supernatant was not immediately recognized as being problematic. It was thought that this supernatant characteristic would only aid in collection of IgM MAb by ammonium sulfate precipitation. The ammonium sulfate precipitation procedure initially used involved a 4°C incubation step after the addition of ammonium sulfate to the desired percentage of saturation. Incubation at 4°C is suggested by Harlow and Lane (1988) and Scott *et al.* (1987) in their precipitation protocols to maximize the amount of MAb collected. However, when using this protocol, MAb collected from different ammonium sulfate fractions demonstrated variable and often high absorbances when tested in wells of the microtiter plate coated with 1% BSA in PBS solution. 1% BSA was used as a competitor conjugate to check for MAb binding to protein which was not hapten specific. It was thought that these background BSA bindings may be the result of irreversible polymerization of the IgM MAb during the cold storage. To overcome this problem new suicide cultures were prepared, and all the supernantants from the suicide cultures, once collected, were immediately processed by a modified ammonium sulfate precipitation procedure without subjection to cold storage. The modified ammonium sulfate precipitation procedure had only a 30 minute, room temperature incubation step to precipitate the IgM MAb, followed by a high gravitational force centrifugation step, dialysis and lyophilization of the MAb. This eliminated any further problems with background binding of the MAb to 1% BSA as a competitor conjugate.

Once the problems with background BSA binding were eliminated, steps were taken to examine the purity and activity of the freeze-dried MAb collected in different ammonium sulfate fractions. Through SDS-PAGE and screening EIAs it was discovered that MAb could be found in all ammonium sulfate fractions from 0 to 80% saturation. However, most of the MAb with the greatest purity could be collected with up to a 60% ammonium sulfate saturation of the supernatant. Figure 4.3 displays a SDS-polyacrylamide gel of supernatant samples from HSFM A1 collected before ammonium sulfate precipitation (preammonium sulfate) and of the 0-60% and 60-80% ammonium sulfate fractions.

From this gel the purity of the 0-60% ammonium sulfate fraction (lane 3) can be observed with the main bands in the lane representing the heavy chains (upper band) and light chains (lower band) of the IgM. Relative to the molecular weight standards (lane 5) the migration of the heavy chain indicated that its size was between the 97.4 kDa and 66 kDa. This molecular weight estimate corresponds to the molecular weight of ~80,000 reported for the heavy chain of the mouse IgM (Goding, 1986). The heavy chain of the

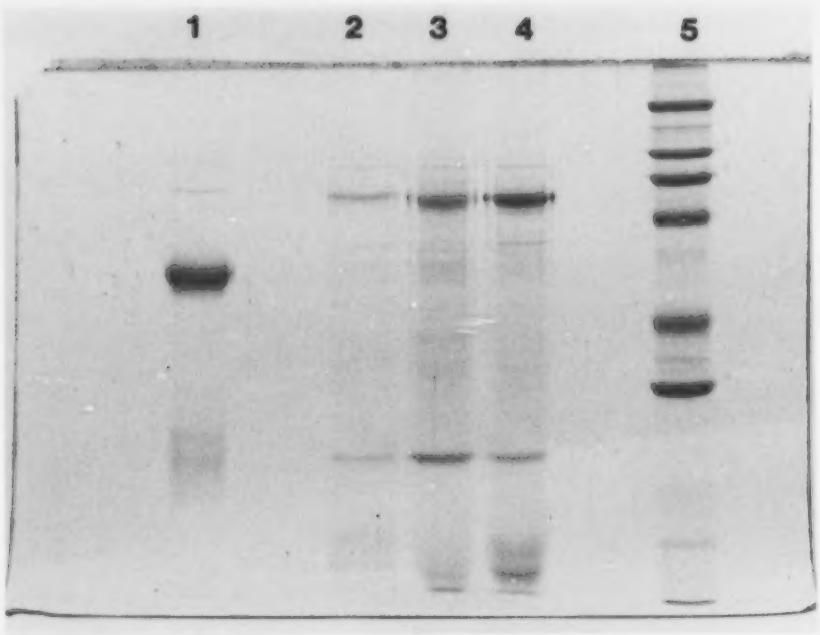


Figure 4.3. SDS-PAGE of Ammonium Sulfate Fractions from HSFM A1 Suicide Culture. Rabbit IgG standard (lane 1), preammonium sulfate precipitation sample (lane 2), 0-60% ammonium sulfate precipitation sample (lane 3), 60-80% ammonium sulfate precipitation sample (lane 4), high molecular weight standards (top to bottom) 205 kDa, 116 kDa, 97,4 kDa, 66 kDa, 45 kDa, and 29 kDa (lane 5).

IgM is also larger than the heavy chain of the rabbit IgG in lane 1. This was expected because the heavy chain of IgG has a mass of ~55 kDa (Harlow and Lane, 1988).The light chains of the IgM and IgG both have approximately the same molecular weight of ~25,000 (Harlow and Lane, 1988; Goding, 1986), and migrated to a similar distance within the gel. The light chain band of the IgG was more diffuse than that of the IgM since the IgG was from a polyclonal source containing light chains of slightly varying structure. An estimate of the molecular weight of the light chain bands could not be made using the molecular weight standards since the smallest standard used was larger than the light chains (29 kDa). The band at the bottom of the gel was probably the J chain of the IgM. The J chain is smaller than the light chain having a molecular weight of ~15,000 (Goding, 1986).

In the 60-80% ammonium sulfate fraction, a protein contaminate corresponding to the molecular weight of the heavy chain of IgM was evident. This protein was discernible because it increased the intensity of the heavy chain versus the light chain in the 60-80% fraction. The contaminating protein was thought to be transferrin a protein additive in the serum-free medium. The molecular weight of transferrin, is reported to be in the range of 76,000 to 81,000 (*The Merck Index*, 1989). Insulin another medium additive (molecular weight of 6000, *The Merck Index*, 1989) was not expected to be found in any of the ammonium sulfate fractions since it should have been removed during the dialysis steps.

It is worthwhile to note that the purity of the preammonium sulfate fraction (lane 2). This sample demonstrated that once the cell debris was removed from the supernatant, the IgM MAb represented the majority of the protein that remained. The ammonium sulfate precipitation procedure was still necessary because it served to further purify and to concentrate the MAb for collection.

IgM MAb Quantification

At different points during the production and purification of the IgM MAb, samples of solutions were taken to determine amount of MAb present. After the the cells had been removed from the suicide culture, supernatant samples were taken prior to ammonium sulfate precipitation (preammonium sulfate). Samples were also taken for analysis from the 0-60% and 60-80% ammonium sulfate fractions after dialysis, and after reconstitution of freeze-dried 0-60% and 60-80% ammonium sulfate fractions.

Both the Bio-Rad protein assay and UV spectrophototmetric analysis were used for IgM MAb determinations for two important reasons. The first reason was that UV spectrophototmetric analysis could not be accurately used on preammonium sulfate supernatants, because the solution was colored due to the presence of phenol red indicator in the original medium. However, it was possible to use the Bio-Rad protein assay on

preammonium sulfate supernatants when values were corrected for the protein content of complete HSFM prior to hybridoma growth. A second reason for determining IgM MAb content in two different manners was the increased confidence it provided in our results. Although both methods were based on spectrophotometric analysis, the source of the chromophores in each case was quite different.

The results shown in Table 4.1 provide a record of MAb collection for three HSFM A1 suicide cultures, using both quantification methods where appropriate. Generally, both the Bio-Rad protein assay and UV spectrophotometric analysis were in agreement as to the amount of IgM MAb present in the samples. However, there were some significant discrepancies such as the protein content of the prefreeze-dried solutions of the 0-60% ammonium sulfate fraction from collections 1 and 2, and in the percentage of MAb in the purified, freeze-dried, and reconstituted material of the 0-60% ammonium sulfate fraction of collection 1, and the 60-80% ammonium sulfate fraction of collection 2. The discrepancies in the MAb content of the prefreeze-dried solutions of the 0-60% ammonium sulfate fractions of collection 1 and 2 resulted in the large differences in the total MAb collected, as determined by the two methods.

When examining only the Bio-Rad protein assay results, it was found that the amount of IgM MAb produced by the hybridomas, prior to purification (preammonium sulfate fraction), ranged from 94 to 130 μg/mL of complete HSFM. This compares favorably with reported production levels of MAb using serum-free media of 50 μg/mL medium (Harlow and Lane, 1988). It may also be observed from Table 4.1 that there was little MAb lost during the purification steps with the exception of collection 2. However, it could not be ascertained whether the loss occurred in the 0-60% or 60-80% ammonium sulfate precipitation steps.

During the determination of the IgM MAb in both the freeze-dried 0-60% and 60-80% ammonium sulfate fractions, it was found that more material was present gravimetrically than could be accounted for using either the UV spectrophotometric determination or Bio-Rad protein assay. Although at a 0.1 M concentration, the ammonium bicarbonate buffer used in the final dialysis was only 1/3 its reported volatile concentration (Perrin and Dempsey, 1974), and tests on buffer alone demonstrated that it was sufficiently volatile to be removed during the 24 h freeze-drying cycle, it was presumed that excess material was still ammonium bicarbonate. A change in buffer behavior in the presence of the IgM MAb may be explained through the association of ammonium and bicarbonate ions with the charged portions of the IgM molecules.

Table 4.1. Determinations by Bio-Rad Protein Assay and UV Spectrophotometric Analysis of the Amount of IgM MAb Collected from Ammonium Sulfate Fractions from HSFM A1 Suicide Cultures

Sample	Method of Determination	MAb Collected from Suicide Culture (mg) Collection 1	Collection 2	Collection 3
MAb in Preammonium Sulfate Fraction	Bio-Rad[a]	12.0 (120 µglmL)[b]	26.2 (131 µg/mL)	9.4 (94 µg/mL)
MAb in 0-60% Ammonium Sulfate Fraction	Bio-Rad	8.96[c](12%)[d]	14.1 (23%)	5.93 (17%)
	UV Spec.	13.8 (20%)	9.52 (23%)	5.61 (17%)
MAb in 60-80% Ammonium Sulfate Fraction	Bio-Rad	2.96[c] (35%)[d]	5.84 (28%)	3.18 (32%)
	UV Spec.	3.53 (31%)	5.53 (40%)	3.95 (37%)
Total MAb (0-60%+60-80%)	BioRad	11.9	19.9	9.11
Total MAb (0-60%+60-80%)	UV Spec.	17.4	15.1	9.56

[a] MAb quantity corrected by subtracting the protein content of complete HSFM (determined by the Bio-Rad protein assay) prior to hybridoma growth

[b] values represent the amount of IgM MAb produced by the hybridoma (µg per mL of supernatant) from the spent suicide culture

[c] total protein content of solution (after second ammonium bicarbonate dialysis, prior to lyophilization)

[d] percentage of protein in reconstituted, freeze-dried material (after second ammonium bicarbonate dialysis)

This would decrease the volatility of the ions during the lyophilization procedure. This was the explanation for the low percentage of recovery of IgM MAb in the purified, lyophilized material after reconstitution.

Although the 60-80% ammonium sulfate fraction was shown to have a higher percentage of MAb in the lyophilized material than the 0-60% ammonium sulfate fraction, the 0-60% fraction was used in further experimentation because of the greater purity of the MAb present. It was thought that the larger amount of ammonium bicarbonate salt present in the 0-60% fraction would not affect the EIA performance and it would aid in dissolving the MAb.

Determination of the Sulfonamide Detection Capabilities of the IgM MAbs

It was only after sufficient IgM MAb was collected from HSFM A1 and A6, and optimization tests were begun, that problems were encountered with the affinities of the IgM MAbs for various competitor conjugates. These problems would later question the potential of the IgM MAbs for sulfonamide detection. The following discussion examines some of the binding capabilities of the MAbs and problems surrounding the use of the MAbs for sulfonamide detection. Although the majority of the testing was performed using IgM MAb purified from HSFM A1, similar results were obtained or expected from HSFM A6.

Problems with OVA-TS as a Competitor Conjugate. The problems associated with background binding to BSA as a coating conjugate were solved with the changes made to the MAb purification procedure. However, early attempts at solving the BSA binding problem raised doubts about the wisdom of using OVA-TS for coating plates. One method previously proposed to overcome the problem of MAb binding to BSA was to replace BSA with a different blocking protein. Because OVA-TS was the competitor conjugate preferred at this time for competitive EIAs, OVA was a logical choice as a blocking protein for the determination of nonspecific binding to the carrier protein. Surprisingly, it was found that the antibody had strong affinity for OVA. However, this was illogical since the mice (to our knowledge) had not been exposed to OVA as an immunogen. In Table 4.2 the affinity of the four competitor conjugates OVA-TS, OVA, 1-BSA and 1% BSA are compared. When first discovered, OVA binding was thought to be the result of the same forces which produced the nonspecific binding observed with BSA and cold precipitation MAb. It was also thought that the binding to OVA would disappear when the problems of BSA binding were solved. However, this was not to be the case. Later, when the cold precipitation problems were eliminated, the MAb still bound strongly to OVA. Since the performance of

Table 4.2. Absorbance Values Obtained using Various Competitor Conjugates and IgM MAb from HSFM A1 Supernatant

Competitor Conjugate	Average Absorbance per Well (450 nm)
OVA-TS (6.35 μg/mL PBS)[a]	1.012 (0.0339)[b]
OVA (57.5 μg/mL PBS)	1.013 (0.0171)
1-BSA (6.6 mg/mL PBS)	1.030 (0.0356)
BSA (10 mg/mL PBS)	0.082 (0.0077)

[a] concentration of competitor conjugate added to each well

[b] standard deviation "s" of average absorbance (n = 6)

OVA-TS in past EIAs was not superior to 1-BSA, and the problem of OVA binding could not be eliminated, competitive indirect EIAs returned to using 1-BSA as the only competitor conjugate for further experimentation.

Problem with PBS/PBST and MAb Binding. During testing of purified MAb from HSFM A1, a problem was noticed with the use of PBS or PBST (PBS/PBST) instead of water as the control solution in the competitive EIA. When performing a competitive EIA, the maximum absorbance values are obtained from wells to which water has been added compared with those which have received an aqueous dilution of sulfonamide or another competitive solution. However, no absorbance was found in wells that had received PBS/PBST as the competitive solution. It was as though PBS/PBST alone, without sulfonamide, was preventing binding or possibly unbinding of MAb on the EIA plate. The results also indicated that the MAb seemed to have a threshold tolerance for PBS/PBST. MAb dissolved in PBS or in 0.05% BSA in PBST still bound to the EIA plate, but when aqueous dilutions of PBS/PBST were used in the competitive EIA, the amount of MAb bound to the plate responded as would be expected for aqueous sulfonamide dilutions (Table 4.3). To evaluate which of the chemicals in PBS may have interfered with MAb binding, solutions of sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, and thimerosal, at their PBS concentrations were substituted for sulfonamide dilutions in a competitive EIA. It was found that only the wells exposed to the sodium chloride solution demonstrated the characteristic PBS reduction in absorbance. A reduced absorbance was not observed in wells that were exposed to PBS concentrations of disodium hydrogen phosphate, potassium dihydrogen phosphate or thimerosal. It was hypothesized that sodium chloride at high enough concentrations could interfere with ionic interactions necessary for the MAb to bind to plate bound antigen.

Sulfonamide Competition. In competitive EIAs performed soon after the fusion, hybridoma supernatants had displayed promising results for the detection of the five sulfonamides SNM, STZ, SMT, SDX, and SQX. Having collected enough purified IgM MAb, and the problems associated with nonspecific binding 1% BSA and PBS/PBST addressed, it was thought that the purified MAb would display similar or greater sulfonamide detection capabilities to those observed in previous competitive EIAs. However, testing with the purified MAb indicated that the five formerly competitive sulfonamides were now not competitive, even at individual sulfonamide concentrations as high as 100 ppm. When repeated testing provided no indication that the purified MAb was detecting sulfonamides within the contraints of the plate bound test, a new definitive experiment was prepared. Using ^{14}CSMT, an experiment was devised which would conclusively prove or disprove whether the MAb was binding to sulfonamide. The use

Table 4.3. EIA Results with PBS and PBST Solutions

Aqueous Dilutions of PBS and PBST (100 µL added per well)	Average Absorbance per Well (450 nm) on 1-BSA Coated Wells
100% PBS[a]	0.055
50% PBS	0.070
10% PBS	0.098
1% PBS	0.103
0.1% PBS	0.106
100% PBST[b]	0.064
50% PBST	0.083
10% PBST	0.106
1% PBST	0.120
0.1% PBST	0.119
control (100 µL water)	0.122[c]

[a] 100% PBS represents the PBS formulation as described under **Reagents** in the EXPERIMENTAL. This was the PBS formulation from which all other dilutions were made.

[b] 100% PBST represents the PBST formulation as described under **Reagents** in the EXPERIMENTAL. This was the PBST formulation from which all other dilutions were made.

[c] average absorbance per well (450 nm) on 1% BSA in PBS coated wells (100 µL water) was 0.055

of ^{14}CSMT was an appropriate choice for the decisive binding experiment, since SMT is a medium sized sulfonamide which has often been reported in the literature as the source of residual sulfonamide contamination (Charm *et al.*, 1988; Singh *et al.*, 1989; Barnes *et al.*, 1990; Larocque *et al.*, 1990; Fischer *et al.*, 1992). Also, the immunogen (1-LPH) was designed with a structure aimed at SMT detection.

The conditions of the MAb-^{14}CSMT binding experiment provided a liquid phase MAb-sulfonamide interaction, which would be free of the constraints involved in the solid-liquid phase interaction of a microtiter plate experiment. Results of the MAb-^{14}CSMT binding experiment (Table 4.4) demonstrated that the MAb did not bind any of the ^{14}CSMT, and less radioactivity was associated with the MAb dialysis fraction (4,291 dpm/mL) than the BSA control fraction (19,045 dpm/mL). This left little doubt about the inability of the MAb to bind sulfonamide.

Ability of MAb to Bind to Hapten of the Immunogen. Realizing that the binding site of the purified IgM MAb was not recognizing the common region of the sulfonamide structure, it was thought that the MAb's affinity might be specific for only the original hapten of the immunogen. Since the overall structure of the original hapten used to immunize the mice differed from any sulfonamide available, a competitive EIA was devised using 1-BSA as both the plate coating conjugate and as the source of competitive hapten. Referring to Figure 4.1, it can be observed that although the carrier proteins differ, 1-BSA has the same hapten and form of conjugation as 1-LPH. Therefore a competitive EIA for the hapten portion of the immunogen can be created using 1-BSA for plate coating and as the source phase of free hapten in solution. If the MAb recognized the original hapten, the 1-BSA in solution should be able to compete equally or better for MAb with plate bound 1-BSA. Experiments indicated that wells of a microtiter plate exposed to a highly concentrated aqueous solution of 1-BSA (1 mg/mL) had approximately 50% less absorbance than wells exposed to water. However, the significance of these results was diminished by the greater than 50% absorbance reduction observed in wells exposed to PBS. This experiment suggested that if the MAb binding site was involved in the attachment to 1-BSA on the plate, it was not indicating any substantial affinity for hapten on the immunogen.

Nonspecific Binding to Test Competitor Conjugates. Although it had been shown that the purified IgM MAb had little or no recognition of sulfonamide or the original immunogen, the fact that the MAb would bind to 1-BSA and not 1% BSA as a competitor conjugate was still perplexing (Table 4.5). Since 1-BSA was only BSA plus conjugated hapten, and it was shown that the MAb seemed to have little if any affinity for the hapten, binding to 1-BSA could only be explained by the MAb recognizing some other

Table 4.4. Results of MAb-^{14}CSMT Binding Experiment

Description of Reactants	Method of Determination	HSFM A1 IgM MAb	BSA
Amount of Freeze-Dried Material (mg)	Measured	0.69[a]	3.02
Amount of ^{14}CSMT Added to Reactants (dpm/mL)[b]	Calculated[c]	904,994	844,660
	Measured	927,202	817,966
Amount of ^{14}CSMT With Even Distribution in Solution (dpm/mL)	Calculated[d]	2,312	2,202
Amount of ^{14}CSMT Inside Membrane Tubing After Dialysis (dpm/mL)	Measured	4,291[e]	19,045[e]
Amount of ^{14}CSMT Outside Membrane Tubing After Dialysis (dpm/mL)	Measured	2,176	2,133

[a] measured value of 0.69 mg of purified IgM based on 3.01 mg of weighed lyophilized material having an MAb concentration of 23%

[b] dpm/mL = decays per minute/milliliter (random counts <0.5%)

[c] calculation based on the addition of a stock ^{14}CSMT containing 6,334,952 dpm/mL

[d] calculation based on the even distribution of ^{14}CSMT inside and outside the membrane tubing without protein binding

[e] values represent the amount of ^{14}CSMT bound (or associated) with the IgM MAb or BSA

Table 4.5. Absorbance Values Obtained for Purified IgM MAb from HSFM A1 using Competitor Conjugates 1-BSA and 1% BSA

Competitor Conjugate	Average Absorbance per Well (450 nm)
1-BSA (2.0 µg/mL PBS)[a]	0.330 (0.0157)[b]
BSA (10 mg/mL PBS)	0.082 (0.0072)

[a] concentration of competitor conjugate added to each well

[b] standard deviation "s" of average absorbance (n = 24)

structural feature of 1-BSA. A possible source of recognition for the MAb was the modified lysine residue through which the hapten was linked to the carrier protein. To test this theory two new competitor conjugates were used.

The first competitor conjugate was a hemisuccinate-linked, sulfamerazine-BSA conjugate (SMR-SA-BSA(H)) whose synthesis was described in chapter 2. In SMR-SA-BSA(H), sulfamerazine was linked to the lysine residues of BSA through the hemisuccinate group attached at the amino-terminal end of sulfamerazine. SMR-SA-BSA(H) was very different from 1-BSA not only in structure and size, but most importantly in that the amino-terminal end of the sulfonamide was involved in a covalent link. The SMR-SA-BSA(H) conjugate had a substitution rate of 22 sulfamerazine molecules per molecule of BSA and its structure is given in Figure 4.1.

The second competitor conjugate was an amide-linked, fumagillin-bovine serum albumin conjugate (BSA-FA) synthesized by Assil and Sporns (1991) for use in a fumagillin EIA. The substitution rate for BSA-FA was 2 fumagillin molecules per molecule BSA and the structure of BSA-FA is given in Figure 4.1. There would be no reason for our MAb to recognize SMR-SA-BSA(H) or BSA-FA conjugates other than the fact that they are conjugates utilizing a hapten-lysine linkage to BSA. Table 4.6 shows the results obtained when 1-BSA, 1% BSA, BSA-FA and SMR-SA-BSA(H) were used as competitor conjugates in a competitive EIA format where water was used as the competitive solution. Finding that the MAb had a greater affinity for BSA-FA and SMR-SA-BSA(H) than for 1-BSA, provided the final evidence leading to the termination of experiments using the purified MAb.

Test of Mouse Bleeds from Second Immunization for Fusion Potential. Having discontinued any further experimentation using IgM MAb from HSFM A1 and A6, efforts were directed towards a possible second fusion using the mice remaining from the first round of immunizations. A probable explanation for the failure of the first fusion was that the mice had not been sufficiently immunized to produce the antibody required for the test. Even though a low affinity MAb would be required for broad spectrum sulfonamide detection, the affinity characteristics displayed by an IgM antibody were obviously less than what was required for the competitive indirect EIA. Also, the problems that were encountered with the IgM in purification and quantitation might not arise if an IgG MAb was produced. Therefore a second set of immunizations was performed with the mice remaining from the first round. The objective of the second immunization was to prepare a population of IgG antibodies with a greater affinity for the hapten on the carrier protein than had previously been achieved. The testing of tail bleeds was also expanded beyond the

Table 4.6. Test for Nonspecific Binding of Purified IgM MAb (HSFM A1) to Various Competitor Conjugates

Competitor Conjugates	Average Absorbance per Well (450 nm)
1-BSA (54.6 μg/mL PBS)[a]	0.244 (0.00841)[b]
BSA (10 mg/mL PBS)	0.082 (0.0029)[c]
BSA-FA (61.6 μg/ mL PBS)	0.358 (0.00968)[c]
SMR-SA-BSA(H) (56.4 μg/mL PBS)	2.430 (0.0322)[b]

[a] concentration of competitor conjugate added to each well

[b] standard deviation "s" of average absorbance (n = 6)

[c] standard deviation "s" of average absorbance (n = 4)

evaluation of simply titer (1-BSA binding) and into the characteristics of nonspecific binding to the conjugates SMR-SA-BSA(H) and BSA-FA, and competition with SQX. SQX was chosen for competition because it was the sulfonamide with the lowest level of detection in the polyclonal EIA developed by Assil *et al.* (1992). Since both mice had responded approximately equally to the immunogen, Table 4.7 contains the results of the test bleed examination for only one mouse receiving a second set of immunizations. The polyclonal serum from each of the mice had affinity for the conjugates SMR-SA-BSA(H), BSA-FA, and at a high concentration of serum (1 in 1000) 1% BSA. Although a small degree of competition was observed at a high SQX concentration (100 ppm), it was believed that another fusion would not likely produce any better results.

Unfortunately, this study concluded without the production of a MAb for multiresidue sulfonamide detection. However, the knowledge that was gained in the areas of MAb production, purification and quantification will aid our laboratory in future experiments in this field.

Table 4.7. Test of Mouse Bleed for Nonspecific Binding and SQX Competition using Various Competitor Conjugates

1000 times dilution of mouse serum with 0.05% BSA in PBST

Competitor Conjugate	Average Absorbance per Well (450 nm): Control Solution (water)[a]	Competitive Solution (100 ppm SQX)[b]
1-BSA (6.83 µg/mL PBS)[c]	2.677 (0.0323)[d]	1.865 (0.0309)
BSA (10 mg/mL PBS)	0.167 (0.0067)	0.163 (0.0035)
BSA-FA (61.6 µg/mL PBS)	1.040 (0.0387)	0.926 (0.0371)
SMR-SA-BSA(H) (56.4 µg/mL PBS)	2.429 (0.0439)	2.276 (0.0253)

10,000 times dilution of mouse serum with 0.05% BSA in PBST

Competitor Conjugate	Average Absorbance per Well (450 nm): Control Solution (water)	Competitive Solution (100 ppm SQX)
1-BSA (6.83 µg/mL PBS)	0.521 (0.0110)	0.223 (0.0211)
BSA (10 mg/mL PBS)	0.068 (0.0066)	0.0687 (0.0040)
BSA-FA (61.6 µg/mL PBS)	0.102 (0.0138)	0.111 (0.0344)
SMR-SA-BSA(H) (56.4 µg/mL PBS)	0.405 (0.0136)	0.370 (0.0324)

[a] 100 µL water added to wells before the addition of diluted serum

[b] 100 µL of a 100 ppm solution of SQX added to the wells before the addition of diluted serum

[c] concentration of competitor conjugate added to each well

[d] standard deviation "s" of average absorbance (n = 3)

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Chapter: 5

CONCLUSION

SUMMARY OF RESULTS

Chapter 2

Chapter 2 of this thesis described the preparation of competitor conjugates for the production of a sulfamerazine (SMR) enzyme immunoassay (EIA). In this chapter many methods were explored to determine hapten-protein molar ratios for the synthesized competitor conjugates. The best methods for the estimation of hapten amounts were a carbon 14 labeled sulfamethazine-bovine serum albumin (^{14}CSMT-BSA) standard curve (for the azo-linked competitor conjugates) and a sulfanilic acid standard curve (for the hemisuccinate-linked competitor conjugates). Confidence in the hapten-protein molar ratios determinations, made by both the ^{14}CSMT-BSA and sulfanilic acid standard curves, were derived from extensive and repeated testing each method underwent. The results of amino acid analysis indicated that it was the histidine residues of bovine serum albumin that were the predominant sites for azo-SMR attachment in the light and medium loaded azo-linked competitor conjugates. Prior to this experiment, it was reported (Sheth *et al.*, 1990) that the tyrosine residues were the primary attachment sites for azo-linked sulfonamide compounds. The weakly basic conditions used in the synthesis of the azo-linked conjugates may have been responsible for the limited participation of tyrosine in the azo-reaction. *N*-chloroacetyl-L-tyrosine/*N*-α-acetyl-L-histidine (NCAT/NAH) standard curves have been commonly used for the determination of hapten-protein molar ratios for azo-linked conjugates (Fenton and Singer, 1971; Dargar *et al.*, 1991; McAdam *et al.*, 1992). This study's attempt at using the NCAT/NAH standard curves demonstrated the lack of wisdom in using methods based on the comparison of absorbance values for an entire protein to that of individual amino acids.

After accurately determining hapten-protein molar ratios for the synthesized competitor conjugates, their performance was evaluated in a competitive indirect EIA. The azo-linked conjugate, SMR-BSA(L) (hapten-protein molar ratio = 4.3) and the hemisuccinate-linked conjugate, SMR-SA-BSA(L) (hapten-protein molar ratio = 6.4) were found to be the best competitor conjugates from each linking method, providing the lowest SMR detection limits. These results were not entirely unexpected. By limiting the amount of plate-bound hapten (accomplished by using a lightly loaded competitor conjugate for plate coating), there is a requirement for less hapten in solution to compete for the hapten-specific antibodies in the antiserum, which results in improved competiton.

Chapter 3

Chapter 3 of this thesis described the evaluation of the competitor conjugates, SMR-BSA(L) and SMR-SA-BSA(L) (synthesized in chapter 2), using milk samples from sulfonamide treated cows. Cows were treated with the sulfonamide, vitamin, and electrolyte preparation, 3-Sulvit. 3-Sulvit was appropriate for this study since it was an over-the-counter medication for cattle, and easily accessible to the dairy farmer. Two commercial tests, the Charm II assay and CITE Sulfa Trio test, were used during the treated cows' milk study, providing a basis against which the performance of the SMR EIAs could be compared. The Charm II assay and CITE Sulfa Trio test were advertised as being able to detect sulfonamides at the 10 ppb level. Recently, a 10 ppb level of detection for sulfonamides residues in cow's milk has become the unwritten standard for commercially produced tests. This has likely resulted from reports that regulatory agencies such as the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) and the United States Food and Drug Administration (FDA) have set low tolerances for sulfonamide residues found in milk (Larocque *et al.*, 1990 and Zomer *et al.*, 1992). The results of our study gave no reason to question the manufacture's claims of detection capabilities, because both the Charm II assay and the CITE Sulfa Trio test performed reasonably well given the probable sulfonamide content of the milk samples. When examined with the commercial tests, the SMR EIAs performed comparably, demonstrating a potential for commercial application. The SMR EIAs were superior to the commercial tests with respect to their ability to provide a quantifiable numerical result as to the amount of SMR present in the milk samples at low ppb levels. The SMR-BSA(L) conjugate had a SMR quantification range from 0.15-5.7 ppb, and the SMR-SA-BSA(L) conjugate had a SMR quantification range from 0.68-16.6 ppb. If another set of experiments was to be proposed for the future evaluation of these competitor conjugates, it would probably involve verifying their detection limits and linear ranges, using chromatographic separation combined with mass spectral analysis.

During the optimization of EIA conditions for the milk study, some unusual characteristics of the SMR-BSA(L) and SMR-SA-BSA(L) competitor conjugates were observed. It was found that SMR-SA-BSA(L) required only half of the coating concentration of SMR-BSA(L) to produce the same amount of absorbance in a well. This indicated that SMR-SA-BSA(L) was binding more antibody than SMR-BSA(L), and this result was unexpected since the immunogen used for antibody production was an azo-linked conjugate. An explanation for this occurrence may have been the added flexibility and extension the hemisuccinate linkage provided hapten on the SMR-SA-BSA(L) competitor conjugate. This may have allowed SMR-SA-BSA(L) to present more hapten to

the solution for antibody binding than was possible for SMR-BSA(L). Also, with the immunogen being an azo-linked conjugate, it was surprising to discover that SMR-BSA(L) had the lower SMR detection limits. This contradicts the theories that the best EIAs are produced with a change in hapten-protein linking methods, which serve to eliminate the undesirable participation of the linking region in the antibody-antigen interaction. The rigidity of the azo-link may serve to weaken the antibody-antigen interaction, decreasing the antigen's (or hapten's) ability to conform to the contours of the antibody's binding site A decrease in the strength of plate-bound antibody-antigen interaction would improve competition in the EIA.

Chapter 4

Although chapter 4 study did not conclude with the preparation of a monoclonal multiresidue sulfonamide EIA, the effort was not without its accomplishments. The experience gained from the problems associated with IgM purfication, in addition to the techniques that were acquired for MAb quantification, will have implications for future hybridoma research performed by myself and others. What should not be overlooked is the complexity of the task that was initially undertaken. It has been less than 20 years since Köhler and Milstein (1975) first introduced the technology of hybridoma production. This project involved one of the most difficult aspects of this technology, the production of an immune response in an animal to a small hapten. Eliciting an immune response to a compound of this small size is sufficiently complicated without imposing constraints on the desired response. The MAb appropriate for our competitive EIA had to be specific enough to recognize only sulfonamides, but not so specific as to limit the number of different sulfonamides which could be detected. Limited success in this endeavor was demonstrated by Sheth and Sporns (1991) and later by Assil *et al.* (1992). However, when Assil *et al.* (1992) isolated the desired population of antibodies from a polyclonal serum by affinity purification, it was found that the subpopulation of antibodies represented only 0.2% of the original serum. It was the very few B cells producing this small fraction of antibodies in the polyclonal serum, that we were attempting to isolate through the production of hybridomas. Chapter 2 demonstrated that it is predominantly the histidine residues on the carrier protein, and not the tyrosine residues, which are involved the hapten-protein azo-linkage. The immunogen 1-LPH was designed with an azo-tyrosine rather than an azo-histidine linkage, which may have contributed to the lack of success in producing the desired MAb. Although it was known from the outset that the production of a MAb with multiresidue sulfonamide specificity was going to be difficult, the rewards of isolating such a MAb would have been well worth the effort.

IMPLICATIONS FOR FUTURE RESEARCH

Production of EIAs for Haptens

The production of an EIA for a hapten represents the most difficult type of EIA to produce. The knowledge gained from the preparation of the SMR EIAs has considerable value in its implications for the production of all hapten based EIAs. It was discovered that hapten-protein linking methods and hapten-protein molar ratios are extremely important for attaining the maximum EIA performance. These two aspects of EIA preparation are often overlooked and sometimes trivialized with respect to all of the other facets involved in EIA production. The detailed characterization of conjugates to be used as immunogens and competitor conjugates should be an integral part of any EIA production.

The conjugate used for immunization and the competitor conjugate used for plate coating are the foundation of the EIA. It should be considered part of the production process of every EIA to try and understand as much as possible about the conjugate being used for immunization, because it represents the template for antibody production. When dealing with the immune system of an animal there are many variables which can not be controlled. It is not necessary to add to the complexity of the process involved in producing antibodies with desired affinities, by not carefully evaluating the nature of the immunogen. The same careful consideration should also be applied to understanding the characteristics of the competitor conjugate, because it provides the basis for EIA competition. It has been demonstrated in this thesis that suitable understanding and manipulation of the competitor conjugate can aid in improving EIA detection levels. However, these considerations seem to have been ignored by other investigators who have developed EIAs.

When first examined, it was surprising to discover how little research had been performed on the evaluation of hapten-protein molar ratio for protein conjugates. After further examination, it was even more surprising to discover how inappropriately the limited research that was available had been applied. There are no universal methods available for the determination of hapten-protein molar ratios. The only accurate methods are based on reaction mechanisms which closely, if not identically, mimic the synthesis conditions of the conjugate being measured. A definite mistake when determining hapten-protein molar ratios, is to attempt to characterize an entire protein based on the reactions of individual amino acids. By no means were the problems related to hapten-protein molar ratio determinations resolved within this thesis. There is still an enormous amount of work required in this area.

When examining the importance of hapten-protein linking methods, the azo-link was shown to be an excellent method of hapten attachment for both the immunogen and competitor conjugate. When utilizing other linking methods for the production of both the immunogen and competitor conjugate, the outcome is often an unsatisfactory EIA. In these EIAs, meaningless levels of competition have resulted from antibodies binding to the hapten-protein linking region. Therefore, in situations where other hapten-protein linking methods may be problematic, the azo-link may provide a viable alternative for EIA production.

The discovery that the hemisuccinate-linked competitor conjugate had the ability to bind more antibody than the azo-linked competitor conjugate may also have implications for future EIA production. The usefulness of polyclonal serum in EIAs is limited by the finite number of antibodies it contains. Extending this limited supply through the use of the appropriate competitor conjugate in an EIA, may be an essential consideration for future EIA development.

A broader range of study of the effects of hapten-protein linking methods and hapten-protein molar ratios, should be considered as an important topic for future research. The significance of this area has been exemplified by a recent decision by the American Chemical Society to start a new journal (Bioconjugate Chemistry) dedicated to increasing the available knowledge in this field. It is hoped that future research will lead to answers to many of the questions which continually arise when hapten-protein linking methods and hapten-protein molar ratios are closely examined.

Sulfonamide EIAs

When sulfonamides were first recognized as a concern in foods, the primary sources were sulfamethazine in pork and milk, and sulfathiazole in honey. Today, the spectrum of animal-based foods with sulfonamide contamination has expanded just as the number of sulfonamides of interest has increased. Almost every animal used in food production seems to be at risk of sulfonamide contamination, and the number of sulfonamides of concern continues to grow every year. Currently, the microbial based multiresidue tests are dominating the sulfonamide test market. However, as the inadequacies of these tests become increasingly evident, immunoassay-based tests should succeed microbial-based tests as the primary methods for sulfonamide testing. In the future the need will be for a commercial sulfonamide tests with not only low detection capabilities, but also with the ability to identify individual sulfonamides. The identification of individual sulfonamides is of considerable importance since if a producer is to be accused of the illegal use of a sulfonamide, it is only sensible to confirm the sulfonamides identity.

Immunoassays are one of the few, if not the only analytical technique, which can identify sulfonamides with great specificity at very low levels. Adding to their attributes was our discovery that the developed SMR EIAs perform better with milk than with water dilutions of SMR. This was a surprising finding because it was presumed that the components found in milk would serve to interfere with and not enhance EIA competition. Further research is required to disclose the nature of this observation. The consequences of such research may substantially influence the future use of immunoassays in the food analysis.

By all indications, immunoassays will remain an important means for food analysis in the future. Whether the enzyme immunoassay format remains the dominant form of immunoassay is of some speculation. Recently, new acoustic, optical and bioelectrochemical detection devices for immune complexes have been developed in the area of immunosensor technology. In the future immunosensors may displace EIA as the major immunoassay format, just as in the past, the EIA displaced the radioimmunoassay.

MAb for Multiresidue Sulfonamide Detection

It would seem that the chances of producing a MAb with multiresidue sulfonamide detection capabilities are small if at all possible. The production of such an antibody goes against the natural evolution of antibodies towards specificity. The possibility of producing a MAb for multiresidue sulfonamide detection might increase if other methods of producing MAb were employed besides the traditional mouse hybridoma. Greater control of MAb specificity may be attainable if methods such as phage or recombinant DNA technology were used for MAb preparation. The use of mice for such a delicate task leaves too many variables to the discretion of the animal's immune system.

Recent literature has seen a surge of interest in the area of serum-free and protein-free media, and soon these media should dominate the area of MAb production. Public opinion has drastically changed towards the use of animals for experimentation. The production of ascitic fluid is probably one of the cruelest forms of experimentation performed on mice. Advances that have been made in serum-free and protein-free media formulations have resulted in increased MAb production rates and decreased production costs. This has made the commercial use of these media a viable alternative to ascites.

The difficulties associated with the production of an IgM, were noted during the course of our MAb investigation. Therefore, it was not surprising to learn from others that IgMs are often discarded in favor of the production of IgG MAbs. IgMs are rejected not only for the prospect of producing higher affinity MAbs, but also to eliminate problems associated with downstream IgM purification.

In the end, it has been said that there is as much to be learned from experiments that do not succeed as ones that do. With respect to all of the research that was involved in preparing this thesis, from the development of a SMR EIA to the attempted production of a multiresidue sulfonamide MAb, much was learned.

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APPENDICES

APPENDIX I

Supplementary Data and Processed Data for Competitive Indirect EIAs using SMR-BSA(L) and SMR-SA-BSA(L) as Competitor Conjugates and Aqueous Dilutions of SMR

Equation used for a best fit, 4-parameter curve: $$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

Competitive Indirect EIA Data obtained using SMR-BSA(L) as the Competitor Conjugate and Aqueous Dilutions of SMR:

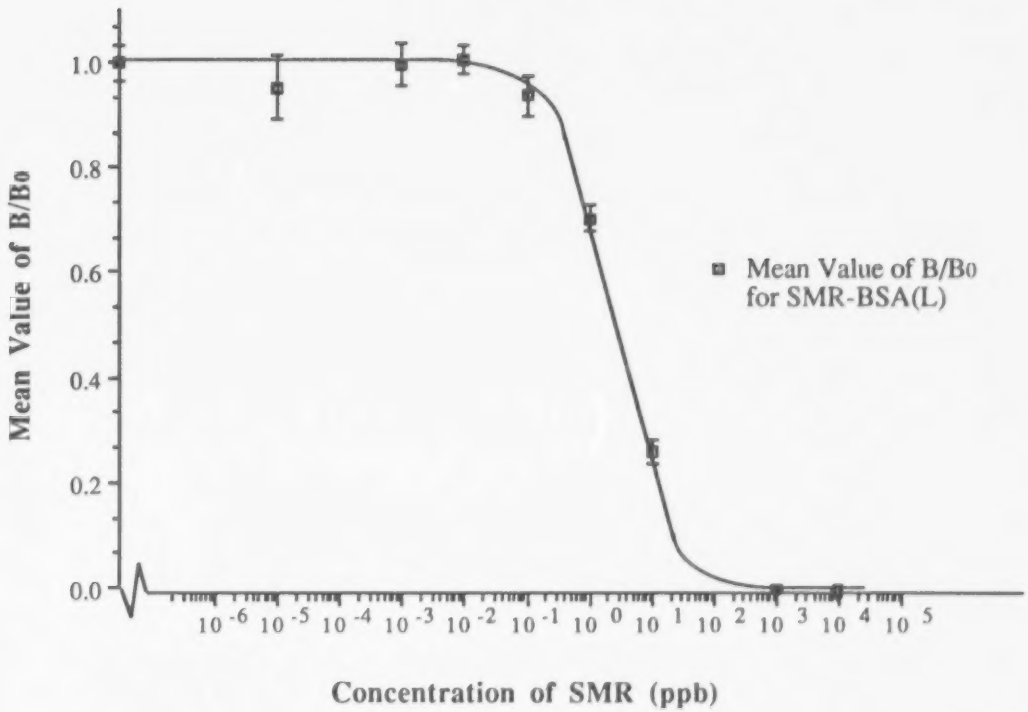
SMR Concentration in parts per billion (x)	B/B_0 for Individual Wells*	Mean of B/B_0 (y)	Standard Deviation of B/B_0
10000	0.001, 0.004, 0.003, 0.003	0.003	0.001
1000	-0.007, -0.007, -0.006, -0.006	-0.006	5.77×10^{-4}
10.00	0.248, 0.255, 0.288, 0.300	0.273	0.025
1.000	0.676, 0.729, 0.741, 0.716	0.715	0.028
0.100	0.912, 0.929, 0.948, 1.006	0.949	0.041
0.010	0.980, 1.049, 1.016, 1.029	1.018	0.029
0.001	0.954, 0.991, 1.050, 1.043	1.009	0.045
1.00×10^{-5}	0.983, 0.950, 1.045, 0.886	0.966	0.066
0.000	0.977, 0.999, 0.968, 0.955, 0.932, 1.012, 1.003, 1.039, 0.976, 1.006, 0.999, 1.045, 1.048, 1.000, 1.050	1.001	0.035

* B = absorbance of a well (450 nm) containing SMR (value of B corrected by deducting a background absorbance value for well)

B_0 = absorbance of a well (450 nm) without SMR (value of B_0 corrected by deducting a background absorbance value for well)

Parameters and Correlation Coefficient for a Sigmoidal Curve (below) generated from the Competitive Indirect EIA Data for SMR-BSA(L) and Aqueous Dilutions of SMR:

a	b	c (I_{50} Value)	d	Correlation Coefficient
0.992	0.976	3.4	-0.00655	0.98


Mean Value of B/Bo
1.0
0.8
0.6
0.4
0.2
0.0
Mean Value of B/Bo for SMR-BSA(L)
10 -6 10 -5 10 -4 10 -3 10 -2 10 -1 10 0 10 1 10 2 10 3 10 4 10 5
Concentration of SMR (ppb)

Representation of the sigmoidal curve generated by SOFTmax software (using the equation for the 4-parameter curve). Data used to produce the sigmoidal curve was obtained from a competitive indirect EIA using SMR-BSA(L) as the competitor conjugate and aqueous dilutions of SMR.

Parameters and Correlation Coefficient generated from the Competitive Indirect EIA Data for SMR-SA-BSA(L) and Aqueous Dilutions of SMR:

a	b	c (I_{50} Value)	d	Correlation Coefficient
0.975	0.919	31	-0.0458	1.00

Competitive Indirect EIA Data obtained using SMR-SA-BSA(L) as the Competitor Conjugate and Aqueous Dilutions of SMR:

SMR Concentration in parts per billion (x)	B/B_0 for Individual Wells*	Mean of B/B_0 (y)	Standard Deviation of B/B_0
1000	-0.005, -0.005, -0.005, -0.007	-0.006	0.001
100.0	0.212, 0.223, 0.213, 0.204	0.213	0.008
10.00	0.700, 0.740, 0.707, 0.714	0.715	0.017
1.000	0.867, 0.895, 0.955, 0.955	0.918	0.044
0.100	0.942, 0.896, 0.964, 0.969	0.943	0.033
0.010	0.931, 0.970, 1.013, 0.993	0.977	0.035
0.001	0.916, 0.955, 1.049, 1.001	0.980	0.058
1.00×10^{-4}	0.937, 0.969, 1.033, 0.981	0.980	0.040
0.000	0.941, 0.952, 1.037, 1.010, 1.005, 1.007, 0.978, 0.961, 0.996, 1.028, 1.019, 1.036, 1.072, 0.967, 1.033, 0.958	1.000	0.037

* B = absorbance of a well (450 nm) containing SMR (value of B corrected by deducting a background absorbance value for well)

B_0 = absorbance of a well (450 nm) without SMR (value of B_0 corrected by deducting a background absorbance value for well)

APPENDIX II

Supplementary Data and Processed Data for Competitive Indirect EIAs using SMR-BSA(L) and SMR-SA-BSA(L) as Competitor Conjugates and Milk Dilutions of SMR

Parameters and Correlation Coefficient generated from the Competitive Indirect EIA Data for SMR-BSA(L) and Milk Dilutions of SMR:

a	b	c (I_{50} Value)	d	Correlation Coefficient
1.00	0.761	0.923	-0.0523	0.99

Parameters and Correlation Coefficient generated from the Competitive Indirect EIA Data for SMR-SA-BSA(L) and Milk Dilutions of SMR:

a	b	c (I_{50} Value)	d	Correlation Coefficient
1.14	0.751	2.11	-0.0112	0.98

Competitive Indirect EIA Data obtained using SMR-BSA(L) as the Competitor Conjugate and Milk Dilutions of SMR:

SMR Concentration in parts per billion (x)	B/B_0 for Individual Wells*	Mean of B/B_0 (y)	Standard Deviation of B/B_0
1000	-0.011, -0.005, 0.011	-0.002	0.011
100.0	0.000, -0.022, 0.011	-0.004	0.017
50.00	-0.011, 0.000, -0.005	-0.005	0.006
10.00	0.076, 0.114, 0.065	0.085	0.026
5.000	0.174, 0.190, 0.212	0.192	0.019
1.000	0.415, 0.527, 0.554	0.511	0.053
0.500	0.505, 0.576, 0.592	0.558	0.046
0.100	0.652, 0.745, 0.701	0.699	0.047
0.050	1.103, 1.065, 0.973	1.047	0.067
0.010	1.005, 0.957, 1.027	0.996	0.036
0.001	1.092, 0.913, 0.989	0.998	0.090
1.00×10^{-4}	0.908, 0.967, 1.005	0.960	0.049
1.00×10^{-5}	0.957, 0.995, 0.891	0.948	0.053
1.00×10^{-6}	1.027, 1.016, 0.918	0.987	0.060
0.000	1.277, 0.880, 0.929, 0.891, 1.027, 1.054, 0.995, 0.853, 0.995, 1.043, 1.141, 1.174, 0.750, 0.875, 1.054	0.996	0.138

* B = absorbance of a well (450 nm) containing SMR (value of B corrected by deducting a background absorbance value for well)

B_0 = absorbance of a well (450 nm) without SMR (value of B_0 corrected by deducting a background absorbance value for well)

Competitive Indirect EIA Data obtained using SMR-SA-BSA(L) as the Competitor Conjugate and Milk Dilutions of SMR:

SMR Concentration in parts per billion (x)	B/B_0 for Individual Wells*	Mean of B/B_0 (y)	Standard Deviation of B/B_0
1.00×10^5	-0.020, -0.010, -0.017	-0.016	0.005
1.00×10^4	-0.023, -0.033, -0.027	-0.028	0.005
1000	-0.013, 0.017, 0.000	0.001	0.015
100.0	-0.010, 0.030, 0.013	0.011	0.020
10.00	0.232, 0.321, 0.225	0.259	0.054
5.000	0.493, 0.626, 0.341	0.487	0.143
1.000	0.685, 0.642, 0.682	0.670	0.024
0.500	0.722, 0.702, 0.917	0.780	0.119
0.100	0.858, 0.980, 0.957	0.932	0.065
0.050	1.159, 1.182, 1.176	1.172	0.012
0.010	1.162, 1.248, 1.291	1.234	0.066
0.001	1.334, 1.192, 1.215	1.247	0.076
1.00×10^{-5}	1.109, 1.176, 1.179	1.155	0.040
1.00×10^{-6}	1.103, 1.169, 1.146	1.139	0.034
1.00×10^{-8}	1.195, 1.079, 1.169	1.148	0.061
0.000	0.911, 0.964, 0.957, 0.897, 1.023, 1.073, 0.957, 1.076, 0.897, 1.076, 1.000, 1.116, 0.957, 1.659, 1.010, 0.964, 1.053, 1.076	1.037	0.169

* B = absorbance of a well (450 nm) containing SMR (value of B corrected by deducting a background absorbance value for well)

B_0 = absorbance of a well (450 nm) without SMR (value of B_0 corrected by deducting a background absorbance value for well)